AD_____

Award Number: DAMD17-02-1-0383

TITLE: Regulation of IAP (Inhibitor of Apoptosis) Gene Expression by the p53 Tumor Suppressor Protein

PRINCIPAL INVESTIGATOR: Maureen E. Murphy, Ph.D.

CONTRACTING ORGANIZATION: Fox Chase Cancer Center
Philadelphia, Pennsylvania 19111

REPORT DATE: May 2005

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	May 2005	Final (1 May 2002 - 30 Apr 2005)

4. TITLE AND SUBTITLE
Regulation of IAP (Inhibitor of Apoptosis) Gene Expression by the p53 Tumor Suppressor Protein

5. FUNDING NUMBERS
DAMD17-02-1-0383

6. AUTHOR(S)
Maureen E. Murphy, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Fox Chase Cancer Center
Philadelphia, Pennsylvania 19111

E-Mail: Maureen.murphy@fccc.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

This research proposal sought to utilize information on p53-dependent programmed cell death toward the design of more effective avenues of breast cancer therapy. Key findings in the past three years included our finding that a common polymorphism in the p53 tumor suppressor protein influences the ability of this protein to induce cell death. Specifically, the arginine 72 form of p53 induces cell death over fifteen-fold better than the proline 72 form. As the latter form is the exclusive form used for gene therapy, these findings have direct implications for this field. We found by mass spectrometry that the arginine 72 form of p53 interacts with the mitochondrial protein BAK. Molecular modeling of the p53-BAK interaction has led to the identification of a "BH3-like" domain in p53 (residues 278-289); mutation of residues in this domain abrogates the ability of p53 to oligomerize BAK. These findings will be extended with the creation of peptidomimetics for this domain of p53, with the goal of effecting increased apoptosis of tumor cells.

14. SUBJECT TERMS
P53, apoptosis, adenovirus, gene therapy, polymorphism

15. NUMBER OF PAGES
31

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Final Progress Report
Department of Defense grant **DAMD17-02-1-0383**
Maureen E. Murphy, Ph.D.

I. Introduction

The goal of this research project is to better understand the tumor suppressive and apoptotic functions of the p53 tumor suppressor protein. As the p53 tumor suppressor gene is mutated or inactivated in up to 30% of breast cancers, understanding how this tumor suppressor gene functions to suppress breast cancer is an important goal. Our research in this application initially proposed creating recombinant viruses that selectively replicated in tumor cells with inactivated or mutant versions of p53. This work led to our surprising discovery that p53 has a transcription-independent mechanism for programmed cell death. Further, this direct, transcription-independent function of p53 is influenced by a common polymorphism in p53, at codon 72. Specifically, we have found that the arginine 72 form of p53 (R72) has over 15-fold increased ability to induce programmed cell death than the proline 72 (P72) form. Significantly, a retrovirus that encodes the R72 form of p53 also has increased ability to target and destroy tumor cells, relative to a retrovirus that expresses equivalent levels of the P72 protein. These studies led to our discovery using mass spectrometry that the R72 form of p53 interacts directly with the pro-apoptotic protein BAK. Research from this proposal has directly led to 3 publications in outstanding peer-reviewed journals, Nature Cell Biology, J Natl Cancer Inst, and Cell Cycle (reprints included in the Appendix). More importantly, the research accomplished by this proposal has the immediate potential to create novel avenues for effecting breast cancer treatment, and for understanding inter-individual differences in cancer risk and treatment efficacy.

II. Body

A. Overview of Progress, Year 3:

Year 3 had two goals. The first was the completion of a selectively-replicating adenovirus in which the adenoviral E1A gene is controlled by the p53-repressible *survivin* promoter. Because such selectively replicating adenoviruses were being rapidly generated by other laboratories, and several of these showed minimal improvement over conventional chemotherapeutic modalities[1], we opted instead for our proposed alternate approach, which was to create adenoviruses that express either the R72 or P72 forms of p53. We proposed to create such viruses, and compare their ability to induce programmed cell death in infected cells. We created the R72 and P72 forms of p53 using the AdEasy vector system from Stratagene. These viruses infect both murine and human cell lines with high efficiency. Our data reproducibly indicate that the adenovirus expressing R72 (Ad-R72) has over 10-fold increased ability to induce programmed cell death, compared to the adenovirus expressing P72 (Ad-P72). These findings suggest that gene therapy with p53 vectors should be used with the R72 version of this protein, instead of the P72 version, which is currently used.

The second goal of year 3 was to elucidate the mechanism underlying transcription-independent cell death by the R72 form of p53. We previously showed in year 2 that the R72 form of p53 induces apoptosis more effectively than P72 because R72 traffics more efficiently to mitochondria, where p53 binds and oligomerizes BAK (Leu et al., Nat Cell Biology; manuscript included in the Appendix). In order to understand this more fully, we narrowed down the region of p53 that is necessary and sufficient to oligomerize BAK to a 20-amino acid peptide from amino acids 270-290. Notably, this region has significant homology to a BH3 domain (BCL2 homology 3, see Figure 2). BH3 domains are known to be capable of directly inducing BAK oligomerization and programmed cell death. These efforts led to our modeling of the p53-BAK interaction using molecular modeling programs. These efforts led to the identification of key residues in p53 responsible for contacting BAK. We fully expect that these efforts will lead to the creation of peptidomimetics with the ability to bind and oligomerize BAK, and subsequently lead to cell death.

B. Detailed Progress:

Task 3. Creation of recombinant adenoviruses expressing either the P72, or R72, forms of p53, and compare these for cell killing ability.

We created the R72 and P72 forms of p53 using the AdEasy vector system from Stratagene. Virus was purified using cesium chloride gradients, and used to infect NIH 3T3 and MCF-7 cells at a multiplicity of infection (m.o.i.) of 1 or 10, and compared to the Ad-GFP control. Infection with the Ad-GFP control at an m.o.i of 10 indicated that 100% of NIH 3T3 cells were infected (not shown). Significantly, cells infected with either Ad-P72, Ad-R72, or Ad-GFP were monitored 24 hours after infection for apoptosis using the Annexin V assay and the Guava Personal Cytometer. These analyses

reproducibly indicated that the Ad-R72 construct induced up to 10-fold more cell death, compared to Ad-P72, despite the fact that nearly equal levels of both proteins were expressed (see Figure 1). Studies are underway to determine if this increase in cell killing by Ad-R72 is true for other breast carcinoma cell lines as well. We are also testing the ability of these adenovirally-expressed p53 proteins to bind and oligomerize BAK in infected cells.

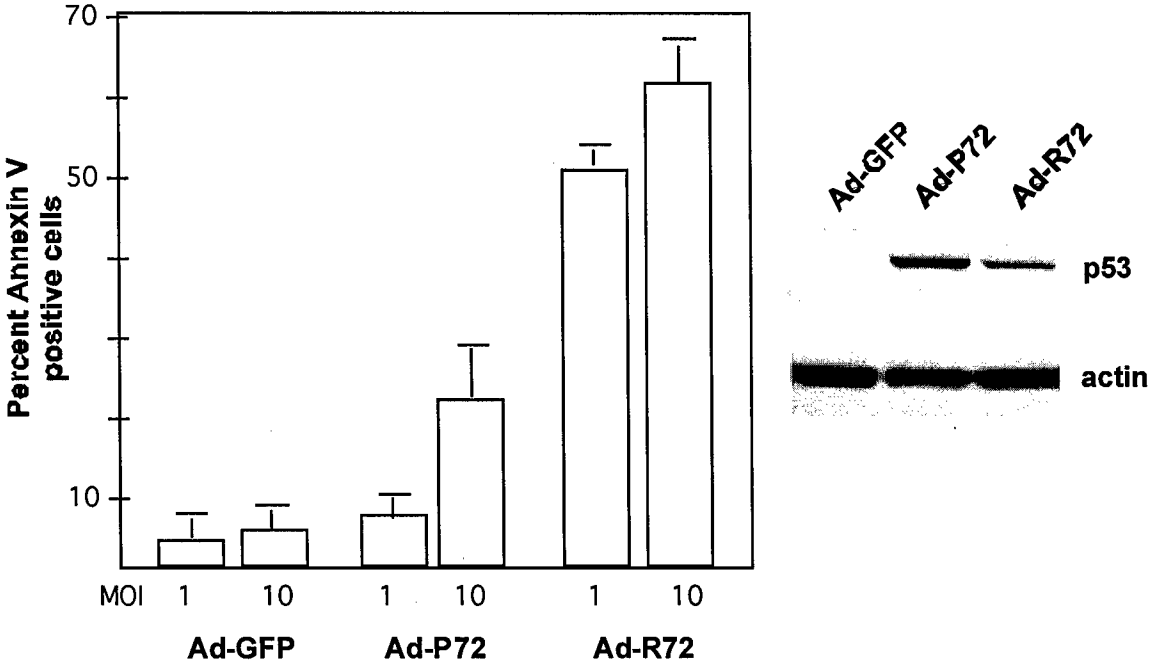

Figure 1. The Ad-R72 virus induces apoptosis up to 10-fold better in NIH 3T3 cells. Left panel: Percent of Annexin positive cells following 24 hours of infection with the MOI shown. Data depicted are the averaged results of three independent experiments, with standard error. Right panel: Level of p53 in infected cells using the antibody Ab-6 (Calbiochem) which detects only human p53.

Task 3: Determine the mechanism for cell death of adenovirally-expressed p53.

We recently reported that p53 binds to the mitochondrial protein BAK, and can directly oligomerize BAK and induce cytochrome c release and cell death[2,3]. In efforts to better understand this activity, we chose to delineate the region of p53 that is necessary and sufficient for interaction with, and oligomerization of, BAK. Using GST fusion proteins containing different portions of p53, and in vitro BAK oligomerization assays, we narrowed down the region of p53 resposnible for binding and oligomerizing BAK to amino acids 270-290 of this protein (not shown). Notably, this region contains significant homology to the BH3 domains of other Bcl2 family members (see Figure 2).

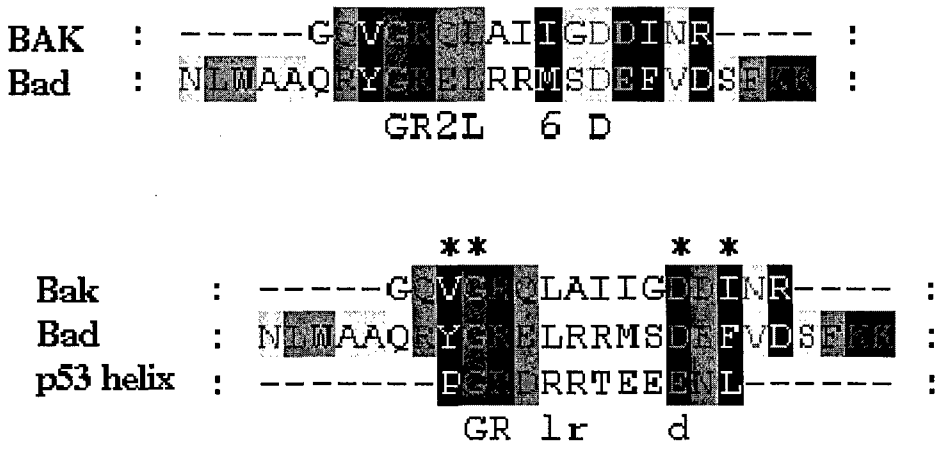

Figure 2. Alignment of the p53 helix from amino acids 278-289 to the BH3 domains from BAK and BAD. Asterisks mark key residues in the alignment.

Molecular modeling of the p53-BAK interaction.

In collaboration with the Molecular Modeling Core Facility at Fox Chase Cancer Center, we recently modeled the interaction of the p53 "BH3-like" domain with BAK (see Figure 3).

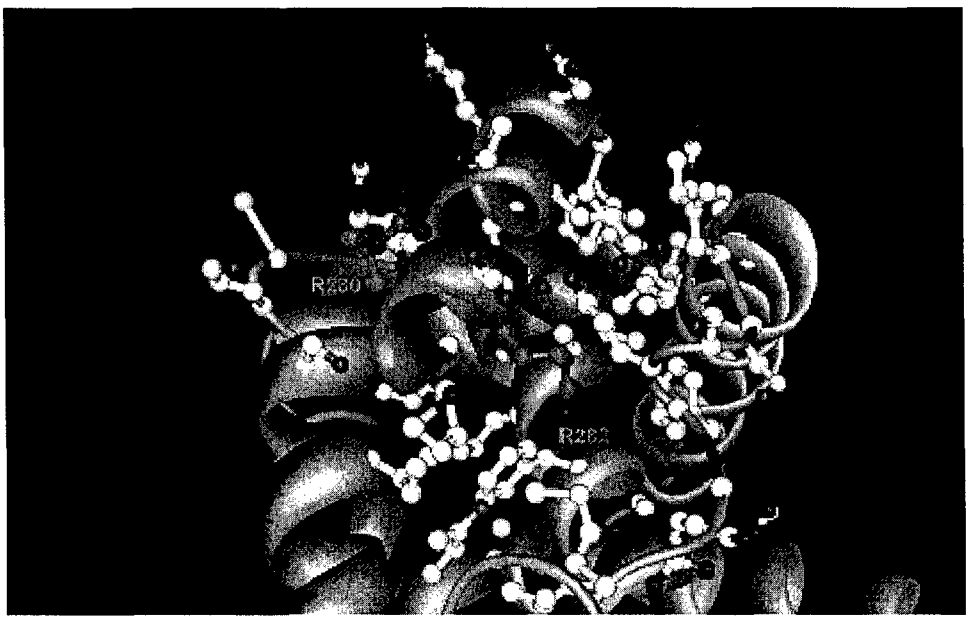

Figure 3. Molecular model of the p53 "BH3-like" domain (amino acids 278-289) with BAK. Key charge-charge interactions at p53 amino acids 280 and 282 are predicted form the model.

This modeling revealed extensive charge-charge interactions between amino acids in p53 and BAK, and highlighted interactions between p53 residues 280 and 282 with

BAK serine 117, and BAK aspartic and glutamic acid residues 85 and 92, respectively. Validation of this model was provided by our finding that mutation of either p53 arginine 280 or 282 to aspartic acid inhibited the ability of p53 to oligomerize BAK (see Figure 4). Interestingly, we also found that mutation of residue 282 to tryptophan, which occurs in a significant percent of human cancers including breast cancers, also abrogates the ability of p53 to oligomerize BAK. We are currently expanding our efforts to validate this model. We plan to extend these studies to test the ability of this 20 amino acid peptide to oligomerize BAK, and induce cell death, in cultured breast carcinoma cell lines.

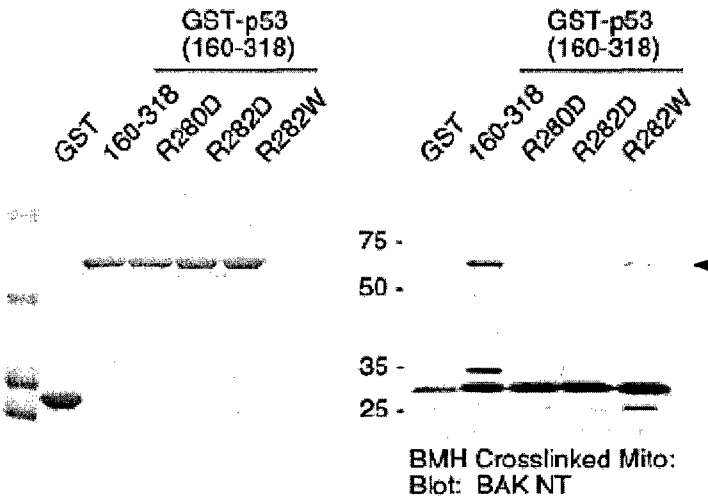

Figure 4. Mutation of p53 residues 280 and 282 results in abrogation of the ability of GST-p53 to oligomerize BAK. In vitro BAK oligomerization analyses were performed with highly purified mitochondria as described in (2). The arrow marks oligomerized BAK. The right panel depicts the purity of purified recombinant p53.

III. Key Research Accomplishments

- Creation of adenovirus vectors that express the arginine 72 polymorphic variant of p53, as well as the proline 72 variant.
- Demonstration that the Ad-R72 virus induces 10-fold increased cell death than the Ad-P72 virus, despite equal levels of protein expressed.
- Molecular modeling of the p53-BAK interaction, which we have shown is critical for transcription-independent programmed cell death by p53.
- Validation of the p53-BAK molecular model, with the demonstration that mutation of two predicted key residues eliminates the ability of p53 to oligomerize BAK.

IV. Reportable Outcomes

Manuscripts, all three years:

1. Bao R, Connolly DC, Murphy M, Green J, Weinstein JK, Pisarcik DA, Hamilton TC. (2002) Activation of cancer-specific gene expression by the survivin promoter. J Natl Cancer Inst. 94:522-8.

2. Leu JI, Dumont P, Hafey M, Murphy ME, George DL (2004). Mitochondrial p53 activates Bak and causes disruption of a Bak-Mcl1 complex. Nat Cell Biol. 6:443-50.

3. Murphy ME, Leu JI, George DL. (2004) p53 moves to mitochondria: a turn on the path to apoptosis. Cell Cycle. 3:836-9.

Viruses:

1. The Ad-R72 and Ad-P72 adenoviruses.

V. Conclusions

- We have shown that the P72 form of p53, which is currently being used in all gene therapy efforts utilizing the p53 tumor suppressor protein, is a significantly poorer inducer of apoptosis than the R72 form of p53.
- We have shown that the R72 form of p53 induces cell death better due to enhanced mitochondrial localization of this form of p53.
- Once at the mitochondria, we used mass spectrometry analysis to identify the BCL2-family member BAK as a key p53-interacting protein.
- We have shown that p53 binds directly to BAK, and can directly induce BAK oligomerization, leading to cytochrome c release and caspase activation.
- We have identified a domain of p53, from amino acids 270-290, that has homology to a BH3 (BCL2-homology 3) domain.
- Mutations in the BH3 domain of p53 abrogate the ability of this protein to bind and oligomerize BAK.

VI. References

1. Post LE. (2002) Selectively replicating adenoviruses for cancer therapy: an update on clinical development. Curr Opin Investig Drugs. 3:1768-72.

2. Leu JI, Dumont P, Hafey M, Murphy ME, George DL (2004). Mitochondrial p53 activates Bak and causes disruption of a Bak-Mcl1 complex. Nat Cell Biol. 6:443-50.

3. Murphy ME, Leu JI, George DL. (2004) p53 moves to mitochondria: a turn on the path to apoptosis. Cell Cycle. 3:836-9.

VII. Bibliography of Publications

1. Bao R, Connolly DC, Murphy M, Green J, Weinstein JK, Pisarcik DA, Hamilton TC. (2002) Activation of cancer-specific gene expression by the survivin promoter. J Natl Cancer Inst. 94:522-8.

2. Leu JI, Dumont P, Hafey M, Murphy ME, George DL (2004). Mitochondrial p53 activates Bak and causes disruption of a Bak-Mcl1 complex. Nat Cell Biol. 6:443-50.

3. Murphy ME, Leu JI, George DL. (2004) p53 moves to mitochondria: a turn on the path to apoptosis. Cell Cycle. 3:836-9.

VIII. List of Personnel

Maureen Murphy, Ph..D., Principal Investigator
Xiaoxian Li, Ph.D., Postdoctoral Associate
Jack Zilfou, Ph.D., Postdoctoral Associate

IX. Appendix Contents

1. Bao R, Connolly DC, Murphy M, Green J, Weinstein JK, Pisarcik DA, Hamilton TC. (2002) Activation of cancer-specific gene expression by the survivin promoter. J Natl Cancer Inst. 94:522-8.

2. Leu JI, Dumont P, Hafey M, Murphy ME, George DL (2004). Mitochondrial p53 activates Bak and causes disruption of a Bak-Mcl1 complex. Nat Cell Biol. 6:443-50.

3. Murphy ME, Leu JI, George DL. (2004) p53 moves to mitochondria: a turn on the path to apoptosis. Cell Cycle. 3:836-9.

Activation of Cancer-Specific Gene Expression by the Survivin Promoter

Rudi Bao, Denise C. Connolly, Maureen Murphy, Jeffrey Green, Jillian K. Weinstein, Debra A. Pisarcik, Thomas C. Hamilton

Background: **Survivin, a member of the IAP (inhibitor of apoptosis) gene family, appears to be overexpressed in common cancers but not in corresponding normal adult tissues. To investigate whether the survivin promoter controls cancer cell–specific gene expression, we determined whether the survivin gene promoter could regulate reporter gene expression in cancer cell lines and xenografts.** ***Methods:*** **Survivin protein levels were determined in human and murine cancer cell lines and in normal tissues of adult C57BL/6 mice by Western blot analysis. A reporter construct in which a portion of the survivin gene promoter was used to drive transcription of a human secreted alkaline phosphatase (SEAP) gene was transiently transfected into cancer cells, and promoter activity was extrapolated from SEAP activity. A2780 human ovarian cancer cells were transfected with this construct, and stable transfectants were injected into the intrabursal ovarian space of immunodeficient mice. Tumor growth was monitored, and plasma SEAP levels were used as a measure of survivin promoter activity *in vivo*.** ***Results:*** **Survivin protein was detected in all cancer cell lines examined but not in most normal adult mouse tissues. After transfection, the survivin promoter was more active in all cancer cell lines than in normal ovarian surface epithelial cells or mouse 3T3 cells. After 0.8×10^6 stable transfectant cells were injected into the intrabursal cavity of mouse ovaries, plasma SEAP activity was detected within 24 hours, and the activity increased with time and tumor growth.** ***Conclusion:*** **Transfection experiments indicate that survivin protein expression in cancer tissue appears to be regulated, at least in part, transcriptionally. Thus, the survivin promoter may be useful in controlling gene expression in cancer cells. [J Natl Cancer Inst 2002;94:522–8]**

Members of the inhibitors of apoptosis (IAP) gene family may play important roles in the survival of cancer cells and the progression of malignancies. The first IAP isolated was the product of a baculovirus gene. Other members of this gene family, including survivin *(1)*, have subsequently been identified in many species, including humans *(2)*. Genes for members of the IAP family are generally characterized by one or more copies of the so-called baculovirus IAP repeat and by a ring finger domain at their carboxyl terminus *(3)*. The survivin gene, located on the long arm of human chromosome 17 at band 25, contains a single baculovirus IAP repeat but no ring finger motif. Because survivin inhibits apoptosis in mammalian cells, the ring finger may not be required for all IAP functions, at least in mammals *(3)*.

Although survivin is not expressed in normal adult human tissues, it is expressed in various human cancers *(4,5)*. Survivin expression may be activated transcriptionally *(6)*; consequently, the survivin promoter might be a cancer-specific promoter with utility in gene therapy or oncolytic viral replication. Such a tumor-selective promoter may also be useful in tumor-prone transgenic mice by activating the expression of a marker gene at the initiation of oncogenesis. In this study, we used transfection experiments to examine whether 1092 base pairs of the 5′ upstream regulatory sequence of the human survivin gene could control the expression of a reporter gene in cancer cell lines derived from tumors of the uterine cervix, breast, ovary, lung, and colon. We also evaluated the activity of the survivin promoter in a cancer cell line *in vivo* as a xenograft.

Materials and Methods

Cell Lines and Cell Culture

The following human cancer cell lines (and their tissue of origin) were used in this study: A2780, OVCAR3, OVCAR5, OVCAR8, OVCAR10, SKOV3, PEO1, and UPN251 (ovary); HT29 (colon); MCF7 (breast); H1299 (lung); and HeLa (uterus). ROSE-TAg is a tumorigenic cell line derived from Fisher 344 rat ovarian surface epithelial (ROSE) cells transformed with simian virus 40 (SV40) large T antigen (TAg) *in vitro*. NuTu19 and NuTu26 are spontaneously transformed cell lines derived from Fisher 344 ROSE cells *(7)*. IG10 and IF5 are spontaneously transformed mouse ovarian surface epithelial (MOSE) cell lines *(8)*. Mc6 is a mammary cancer cell line derived from a mammary tumor of a mammary tumor-prone C3 (1)/TAg transgenic mouse line, and Pr14 is a prostate cancer cell line derived from a prostate tumor of a prostate cancer-prone line of C3 (1)/TAg transgenic mice *(9,10)*. Normal MOSE cells were isolated from the ovaries of C57BL/6 adult mice *(8)* and used for up to three passages. Normal ROSE cells were isolated from the ovaries of Fisher 344 rats *(7)* and used for up to five passages. Normal human ovarian surface epithelial (HOSE) cells were derived from normal human ovaries as previously described *(11)*. NIH 3T3 is an immortalized, nontumorigenic mouse fibroblast cell line. All cell lines were maintained at 37 °C in a humidified incubator with an atmosphere of 5% CO_2/95% air. HOSE, A2780, OVCAR3, OVCAR5, OVCAR8, OVCAR10, SKOV3, PEO1, UPN251, ROSE-TAg, NuTu19, NuTu26, MCF7, and normal ROSE cells were cultured in RPMI 1640 medium (GIBCO BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS). IG10, IF5, Mc6, Pr14, and MOSE cells were cultured in Dulbecco's modified Eagle medium (DMEM; GIBCO BRL) plus 5% FBS. NIH 3T3 cells were cultured in

Affiliations of authors: R. Bao, D. C. Connolly, J. K. Weinstein, D. A. Pisarcik, T. C. Hamilton, (Ovarian Cancer Program), M. Murphy (Department of Pharmacology), Fox Chase Cancer Center, Philadelphia, PA; J. Green, Laboratory of Cell Regulation and Carcinogenesis, National Cancer Institute, Bethesda, MD.

Correspondence to: Thomas C. Hamilton, Ph.D., Ovarian Cancer Program, Fox Chase Cancer Center, 7701 Burholme Ave., Philadelphia, PA 19111 (tc_hamilton@fccc.edu).

See "Notes" following "References."

DMEM plus 10% calf serum, and H1299 and HeLa cells were cultured in DMEM plus 10% FBS. All media were supplemented with streptomycin (100 μg/mL), penicillin (100 U/mL), glutamine (0.3 mg/mL), and pork insulin (0.25 U/mL or 1× ITS [insulin, transferrin, and selenium]; GIBCO BRL, Rockville, MD).

Western Blot Analysis

At about 70% confluence, cells were harvested with trypsin/EDTA, and PBS-washed cell pellets were stored at –70 °C until use. Whole-cell protein was extracted from the cell pellets with RIPA buffer (50 m*M* Tris–HCl at pH 7.4, 150 m*M* NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate, and 1% sodium deoxycholate). Protein was also extracted from normal tissues (brain, thymus, heart, lung, liver, stomach, small intestine, bladder, kidney, ovary, oviduct, uterus, spleen, pancreas, and skeletal muscle) of female C57BL/6 mice (8 weeks, 12 months, or 16 months of age). Tissues were homogenized in a Mini Bead Beater (BioSpec Products, Inc., Bartlesville, OK) and the T-MER tissue protein extraction reagent (Pierce, Rockford, IL). For survivin detection, 30 μg of total protein extract was resolved on sodium dodecyl sulfate–15% polyacrylamide gels and transferred to nitrocellulose membranes (Amersham, Piscataway, NJ). The blots were hybridized with an anti-survivin polyclonal antibody (diluted 1:2000; Novus Biological, Littleton, CO), followed by incubation with a peroxidase-conjugated goat anti-rabbit second antibody (diluted 1:5000; Amersham). The peroxidase activity was detected by the ECL method (NEN, Boston, MA).

Vector Construction

A 1092-base-pair fragment of the human survivin gene promoter (nucleotides 1821–2912, GenBank accession number U75285) was excised from plasmid SpI with restriction enzymes *Kpn*I and *Xho*I *(12)*. The secreted alkaline phosphatase (SEAP) expression vector under control of the survivin promoter (pSRVN-SEAP) was constructed by subcloning the *Kpn*I-*Xho*I fragment into the multiple cloning site of the SEAP expression vector pSEAP-Basic (Clontech, Palo Alto, CA). To generate stable transfectants, the pSRVN-SEAP-NEO plasmid was constructed by subcloning the SRVN-SEAP sequence (a *Kpn*I-*Xba*I fragment) from pSRVN-SEAP into the PC3 vector *(13)*, a modified pcDNA3 vector (Invitrogen, San Diego, CA) without the cytomegalovirus promoter.

Transient Transfection

The pSRVN-SEAP plasmid was transiently transfected into cell lines by use of the TransIT-LT1 transfection reagent (Pan-Vera, Madison, WI). Briefly, 3×10^5 cells were placed into each well of a six-well plate in 2 mL of complete medium. After incubation overnight, cells were 40%–50% confluent, and a mixture of 2 μg of pSRVN-SEAP plasmid, 0.2 μg of pGL3 control plasmid, 6 μL of LT1 transfection reagent, and 100 μL of serum-free medium was added to each well. The pGL3 control plasmid (Promega, Madison, WI), which is a luciferase expression vector driven by the SV40 promoter, was used to assess transfection efficiency and, hence, normalize each transfection. Two other plasmids, pSEAP-Basic (a promoterless SEAP construct) and pSV40-SEAP (a SEAP expression vector with the SV40 promoter) (Clontech), were also used for each cell line as negative and positive controls, respectively. Medium (100 μL) was removed 48 hours after transfection and used to determine SEAP activity after normalization of the transfection efficiency. Briefly, the adherent cells were washed once with PBS, exposed to 1 mL of lysis buffer (Promega), and scraped from dishes with a cell scraper. After centrifugation of the cell lysates at 15700 relative centrifugal force (rcf) for 1 minute, the supernatants were removed and stored at –70 °C until luciferase activity was assayed. Luciferase activity was determined by mixing 5 μL of supernatant with 100 μL of luciferase assay reagent (Promega) and determining the relative luminescence with a luminometer (Analytical Luminescence Laboratory, San Diego, CA). This procedure allowed us to adjust the amount of conditioned medium used to allow for differences in transfection efficiency.

Stable Transfection

The pSRVN-SEAP-NEO plasmid was linearized with restriction enzyme *Pvu*I and purified by phenol–chloroform extraction and ethanol precipitation. Before electroporation, subconfluent A2780 cells were trypsinized, washed twice with PBS, and resuspended at 10×10^6 cells in 0.7 mL of PBS. The cell suspension was transferred into a Gene Pulser cuvette (Bio-Rad Laboratories, Hercules, CA), and 5 μg of linearized pSRVN-SEAP-NEO or control vector PC3 was added. After 10 minutes on ice, the cells were subjected to electroporation by using the Gene Pulser II System (Bio-Rad Laboratories) at 250 V/cm and 975 μF and then plated in three 10-mm Petri dishes with complete medium. One day later, medium was changed to complete growth medium supplemented with G418 at 500 μg/mL. After 2 weeks, the G418-resistant clones were isolated with cloning cylinders. SEAP activity in the conditioned medium from individual clones was determined when the cells were nearly confluent.

Animal Study

Female CB17/ICR SCID (severe combined immunodeficient) mice, approximately 8 weeks of age and weighing approximately 20 g, were used to establish orthotopic ovarian tumors. All these mice were bred in the Laboratory Animal Facility at Fox Chase Cancer Center, were maintained in specific pathogen-free conditions, and received commercial food and water *ad libitum*. Institution guidelines were followed in handling the animals. To establish the orthotopic tumors, cultured A2780 transfectants (two SRVN-SEAP-NEO clones, $A2780^{SSN1}$ and $A2780^{SSN2}$, and one vector control clone, $A2780^{PC3}$) were harvested with 0.05% trypsin-EDTA (GIBCO BRL), washed in PBS, and resuspended in RPMI-1640 complete medium at 40×10^6 cells per milliliter. Before intrabursal implantation of tumor cells, eight SCID mice were anesthetized with a 15:3:5:152 mixture of ketamine-HCl (100 mg/mL), acepromazine malleate (10 mg/mL), xylazine hydrochloride (20 mg/mL) (Fort Dodge Animal Health, Fort Dodge, IA), and 0.9% normal saline, injected intraperitoneally at 10 μL/g of body weight. The skin was disinfected with Wescodyne and 70% ethanol. A small incision was made on one side of the back to locate the ovary. The oviduct was held with small forceps, and a 26-gauge needle connected to a syringe was inserted into the oviduct and was passed through the infundibulum until the needle tip reached the space between the bursa and the ovary. Approximately 20 μL of the cell suspension (about 0.8×10^6 cells) was injected into the intrabursal space. The needle was slowly removed, the ovary

was replaced in the abdominal cavity, and the body wall was closed with sutures. One ovary of each animal was injected.

Plasma for SEAP analysis was obtained by orbital puncture with heparinized glass tubes (Fisher Scientific, Pittsburgh, PA) on days 0, 1, 3, 6, and 9 after cell implantation. About 20 μL of plasma was obtained after the blood was centrifuged at 4500 rcf for 7 minutes *(14)*. Animals were sacrificed 14 days after implantation; ovaries were removed, embedded in paraffin, and sectioned for histopathologic analysis.

SEAP Assay

SEAP activity in culture medium or plasma was determined by a chemiluminescence or fluorescence method using Great Escape SEAP kits from Clontech *(15)*. In brief, 5-μL samples were mixed with 45 μL of dilution buffer and incubated in a oven at 70 °C for 45 minutes. Sixty microliters of assay buffer containing L-homoarginine was then added. After a 5-minute incubation at room temperature, the samples were exposed to 60 μL of chemiluminescent substrate CSPD (disodium 3-[4-methoxyspiro{1.2-dioxetane-3,2′-(5′-chloro)tricyclo(3.3.1.1)-decan}4-yl]phenyl phosphate) (1.25 mM) or 3 μL of fluorescent substrate 4-methylumbelliferyl phosphate (Clontech). Chemiluminescence was measured with a luminometer (Analytical Luminescence System) after a 10-minute incubation at room temperature.

After a 60-minute incubation in the dark, fluorescence was measured with a CytoFluor II fluorometer (Bio-Rad Laboratories) with excitation and emission wavelengths of 360 nm and 449 nm, respectively. SEAP activity was determined from a standard curve.

To determine whether exogenous SEAP could be separated from endogenous placental alkaline phosphatase of pregnant animals, plasma from two C57BL/6 pregnant mice at embryonic day 12, one normal control mouse, and one CB17/ICR SCID mouse carrying an A2780^{SEAP13} cell implant *(14)*, was isolated. Five microliters of plasma was mixed with 45 μL of dilution buffer, and the mixture was heated to 70 °C for 0, 20, 40, or 60 minutes. Alkaline phosphatase activity was determined as described above.

Results

Survivin Protein Levels in Cancer Cell Lines, Normal Cells, and Normal Tissues

To determine how frequently the increased expression of survivin is detected in cancer cell lines compared with normal tissues and cells, protein extracts were prepared from cells and tissues derived from different species and subjected to western blot analysis. As shown in Fig. 1, A, survivin protein was detected as an intense band at 16.5 kDa in all the human ovarian cancer cell lines, including A2780, OVCAR3, OVCAR5,

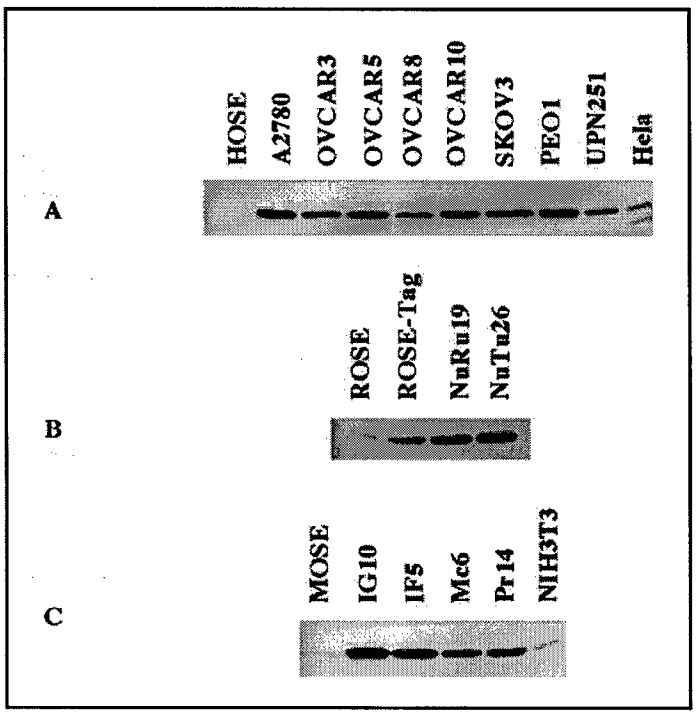

Fig. 1. Western blot analysis of survivin protein in cell lines from various species. **A)** Survivin protein level in human ovarian cancer cell lines (A2780, OVCAR3, OVCAR5, OVCAR8, OVCAR10, SKOV3, PEO1, and UPN241) was compared with that in normal human ovarian surface epithelial (HOSE) cells. The survivin-expressing cell line HeLa from uterine cervix was the positive control. **B)** Survivin protein level in transformed rat ovarian surface epithelial cell lines (ROSE-Tag, NuRu19, and NuRu26) was compared with that in early-passage rat ovarian surface epithelial (ROSE) cell lines. **C)** Survivin protein level in transformed mouse ovarian surface epithelial cell lines (IG10 and IF5), mammary (Mc6) and prostate (Pr14) tumor cell lines from transgenic mice, and a mouse fibroblast cell line (NIH 3T3) was compared with that in early-passage mouse ovarian surface epithelial (MOSE) cells.

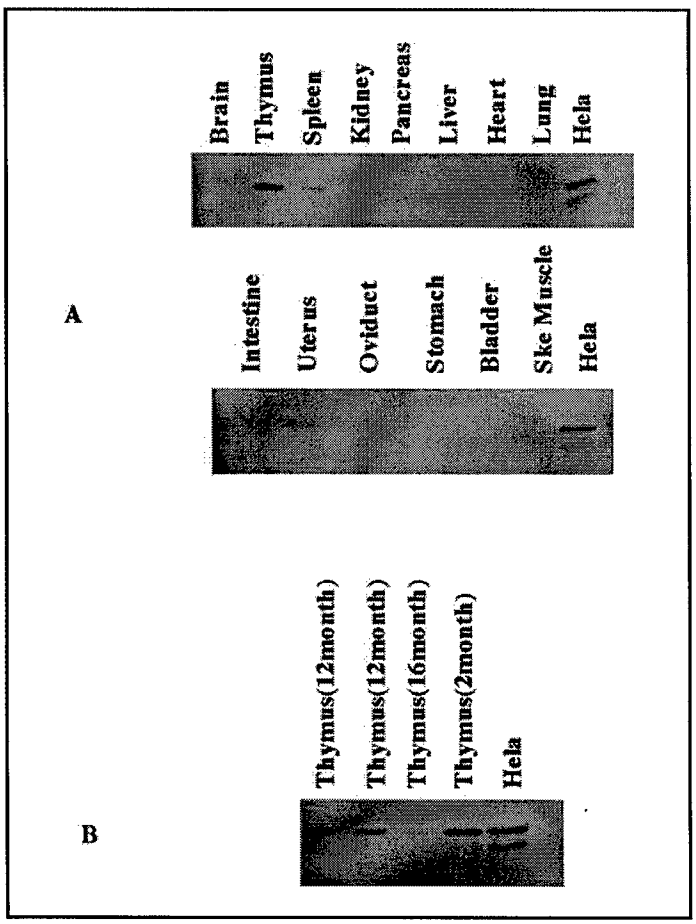

Fig. 2. Western blot analysis of survivin protein in tissues from C57BL/6 mice as indicated. The uterine cervix cancer cell line HeLa was used as the positive control. **A)** Survivin protein level in tissues of 8-week-old C57BL/6 mice. **B)** Survivin protein level in thymus from mice aged 2, 12, and 16 months. Ske = skeletal.

OVCAR8, OVCAR10, SKOV3, PEO1, and UPN251, but not in the normal HOSE cells. An intense 16.5-kDa survivin band was also detected in transformed rat ovarian surface epithelial cell lines (ROSE-TAg, NuTu19, and NuTu26), but only a faint band was detected in early-passage normal ROSE cells (Fig. 1, B). Consistent with our findings in normal and transformed ROSE cells, an intense survivin band was detected in the transformed MOSE cell lines (IG10 and IF5), but only a faint band was detected in the normal MOSE cells (Fig. 1, C). An intense survivin band was also detected in tumor cell lines from transgenic mice prone to develop mammary tumors (Mc6) *(9)* or prostate tumors (Pr14) *(10)*. A faint survivin band was detected in nontumorigenic mouse fibroblast cell line NIH 3T3 (Fig. 1, C).

Survivin Protein Levels in Normal Adult Mouse Tissues

Levels of survivin protein in normal tissues from 8-week-old female C57BL/6 mice were determined by western blot analysis. No survivin protein was detected in brain, heart, lung, liver, stomach, intestine, bladder, kidney, ovary, oviduct, uterus, pancreas, or skeletal muscle. Survivin was detected in the thymus and, to a lesser extent, in the spleen (Fig. 2, A). Because age-associated thymic atrophy could result in a decrease in survivin protein in the thymus, we evaluated survivin expression in 12-month-old and 16-month-old C57BL/6 mice and detected a marked reduction of survivin protein in mature, as opposed to young adult, mice (Fig. 2, B).

In Vitro Survivin Promoter Activity

We constructed the pSRVN-SEAP plasmid to determine whether the survivin promoter functioned in cancer cells. Promoter activity was determined from the SEAP activity in conditioned medium from transiently transfected cells. In A2780 cells transfected with the promoterless pSEAP-Basic plasmid, SEAP expression was almost baseline (Table 1). In several other cancer cell lines, SEAP expression was also almost baseline, but in others, the promoterless plasmid had some activity. In all cancer cell lines transfected with a plasmid containing the survivin promoter (i.e., pSRVN-SEAP), SEAP expression was five-fold to about 400-fold higher than that observed with the promoterless pSEAP-Basic plasmid (Table 1; Fig. 3, A). However, early-passage normal ROSE and MOSE cells similarly transfected showed less SEAP expression when transfected with pSRVN-SEAP than with pSEAP-Basic (Table 1). To determine the relative promoter activity of the survivin promoter compared with the relatively strong SV40 viral promoter, we transfected the various cell lines with the SV40 promoter-driven SEAP expression plasmid pSV40-SEAP and measured SEAP expression. The survivin promoter was more active in the cancer cell lines, and the SV40 promoter was more active in the nontransformed cell lines (i.e., NIH 3T3, ROSE, and MOSE cells) (Table 1; Fig. 3).

In Vivo Survivin Promoter Activity

To determine whether the survivin promoter could induce enough SEAP activity to monitor tumor growth *in vivo*, we created stable A2780 transfectants harboring stably integrated SRVN-SEAP-NEO. Two clones ($A2780^{SSN1}$ and $A2780^{SSN2}$) were selected because of their relatively high SEAP production (Fig. 4, A). These two SRVN-SEAP-NEO clones and one vector control clone ($A2780^{PC3}$) were used to generate orthotopic ovarian tumors by injection into the intrabursal space of mouse ovaries to mimic early ovarian cancer. After tumor cell implantation, plasma was collected at designated intervals to measure SEAP activity. SEAP activity was detected as early as 24 hours in animals implanted with 0.8×10^6 cells from either of the two SRVN-SEAP-NEO clones and increased with time and tumor growth. In contrast, SEAP activity was not detected in the animal injected with the vector control clone (Fig. 4, B and C). Paraffin sections prepared from ovaries removed on day 14 had small tumors in the intrabursal cavity in all mice injected with a pSRVN-SEAP-NEO clone ($A2780^{SSN}$) or the vector control clone ($A2780^{PC3}$) (Fig. 4, D). Contralateral ovaries were normal.

Sensitivity of Endogenous and Exogenous Alkaline Phosphatase to Heat Treatment

To determine whether endogenous alkaline phosphatase activity could be separated from transgenic SEAP activity, we used heat to inactivate the endogenous activity. Plasma from normal control mice, pregnant mice, and a mouse carrying $A2780^{SEAP13}$ cells *(14)* was treated with heat for 0, 20, 40, or 60 minutes, and

Table 1. Activity of survivin promoter relative to a promoterless or simian virus 40 promoter-driven secreted alkaline phosphatase plasmid in cell lines with various origins*

Cell type	pSEAP-Basic (1)	pSRVN-SEAP (2)	pSV40-SEAP (3)	Ratio (2)/(1)	Ratio (2)/(3)
A2780	100 ± 20	16 000 ± 900	5400 ± 400	160	3
$OVCAR3^s$	5000 ± 600	67 000 ± 12 000	40 000 ± 1100	13	2
SKOV3	80 ± 30	41 000 ± 1800	20 000 ± 1300	513	2
NuTu19	20 ± 1	5500 ± 400	3100 ± 200	275	2
IF5	200 ± 20	3000 ± 900	1400 ± 100	15	2
IG10	200 ± 10	1200 ± 30	600 ± 20	6	2
Mc6	300 ± 40	11 000 ± 800	4300 ± 300	37	3
Pr14	50 ± 0	1000 ± 10	600 ± 30	20	2
$MCF7^s$	1500 ± 200	160 000 ± 4500	84 000 ± 5600	107	2
$HeLa^s$	43 000 ± 3700	8 800 000 ± 17 000	4 000 000 ± 230 000	204	2
$HT29^s$	21 000 ± 400	8 800 000 ± 6300	4 600 000 ± 48 100	409	2
$H1299^s$	95 000 ± 1200	8 400 000 ± 250 000	4 200 000 ± 110 000	90	2
NIH 3T3	80 ± 20	600 ± 50	1100 ± 200	8	<1
$ROSE^s$	15 000 ± 3700	8100 ± 2700	26 000 ± 2300	<1	<1
$MOSE^s$	14 000 ± 2000	110 000 ± 18 000	430 000 ± 47 000	8	<1

*Secreted alkaline phosphatase analyses (indicated with a superscripted "s") were done by the luminescence method and expressed as relative luminescence units. Other analyses were done by the fluorescence method and expressed as relative fluorescence units. ROSE = rat ovarian surface epithelial; MOSE = mouse ovarian surface epithelial; SEAP = selected alkaline phosphatase; SV40 = simian virus 40.

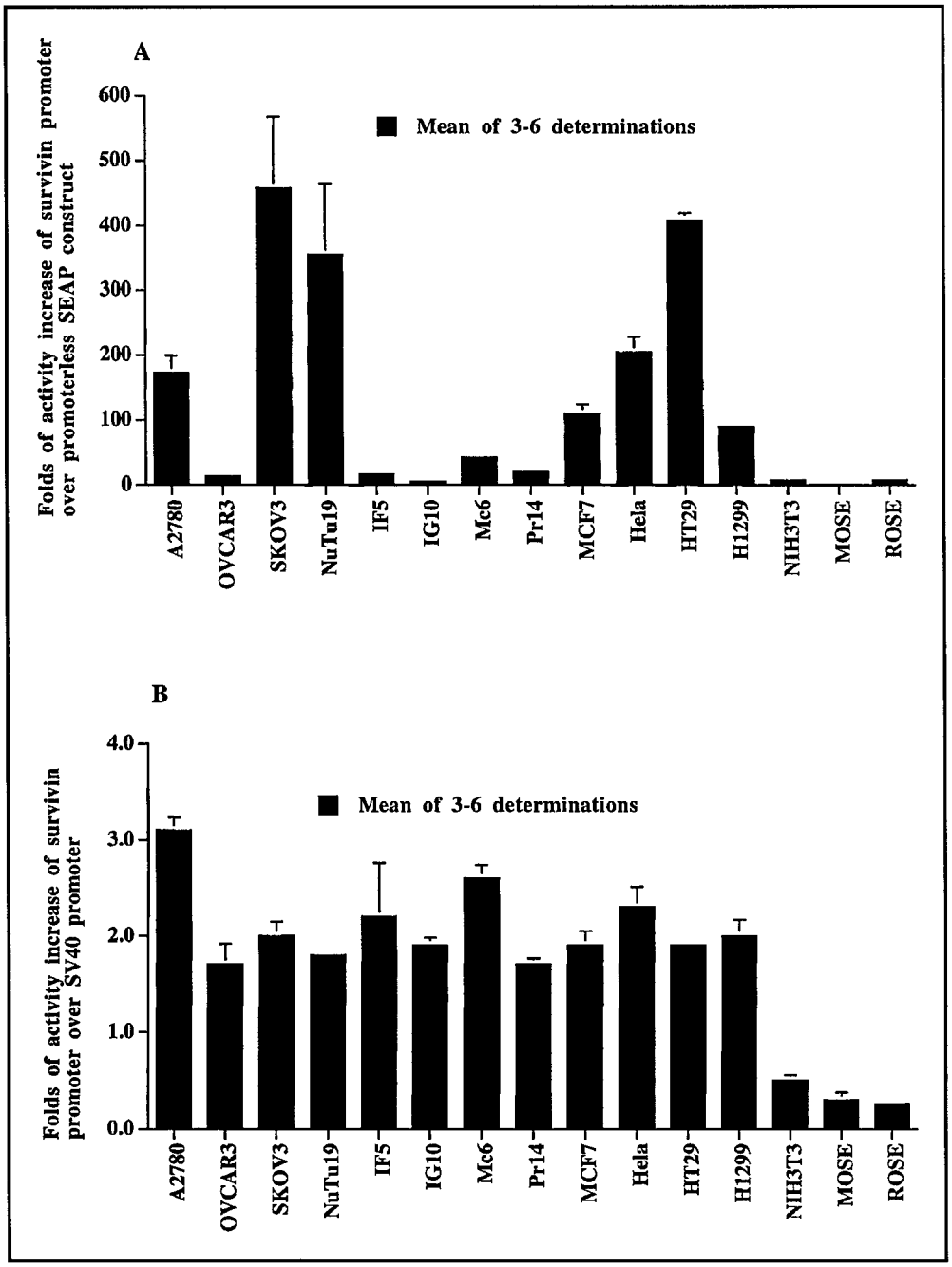

Fig. 3. Activity of the survivin promoter in cell lines as indicated. **A)** Activity of the survivin promoter relative to the activity of the promoterless secreted alkaline phosphatase (SEAP) construct. Activity of the promoterless SEAP construct was defined as 1. **B)** Activity of the survivin promoter relative to the activity of the simian virus 40 (SV40) promoter. Activity of the SV40 promoter was defined as 1. Rose = rat ovarian surface epithelial; MOSE = mouse ovarian surface epithelial. All values are the mean of three to six determinations. **Error bars** are 95% confidence intervals.

alkaline phosphatase activity was determined. As shown in Fig. 5, alkaline phosphatase activity in plasma of normal and pregnant mice decreased quickly after the heat treatment at 70 °C and was still low 40 minutes later. However, plasma alkaline phosphatase activity in the mouse xenografted with $A2780^{SEAP13}$ cells had not decreased after 60 minutes of heat treatment. Therefore, exogenous SEAP activity can be monitored during tumor development and effectively separated from endogenous alkaline phosphatase activity by heat treatment.

Discussion

Regulated induction of apoptosis preserves normal homeostasis and organ morphogenesis. Aberration of this process may contribute to cancer development by prolonging cell viability. Members of the IAP gene family have emerged as unique modulators of apoptosis, possibly by the direct inhibition of terminal effector caspases 3, 7, and 9. Survivin, a new member of the human IAP family, was identified by hybridization screening of human genomic libraries with the complementrary DNA for effector cell protease receptor-1, a factor Xa receptor *(1)*. Unlike other IAP family members, survivin contains a single baculovirus IAP repeat and no carboxyl-terminal ring finger region. Most importantly, at variance with other IAPs such as BCL2, which is present in both normal and transformed cell types, survivin was originally reported to be completely undetectable in normal human adult tissues but expressed during fetal development *(1,16,17)*. Our data on the mouse (this report) and in a published report *(18)*, however, indicate that survivin is present in the thymus and spleen of young adult mice. It seemed unlikely that this difference was related to differences between survivin promoters in mice and humans, because the homology in this region

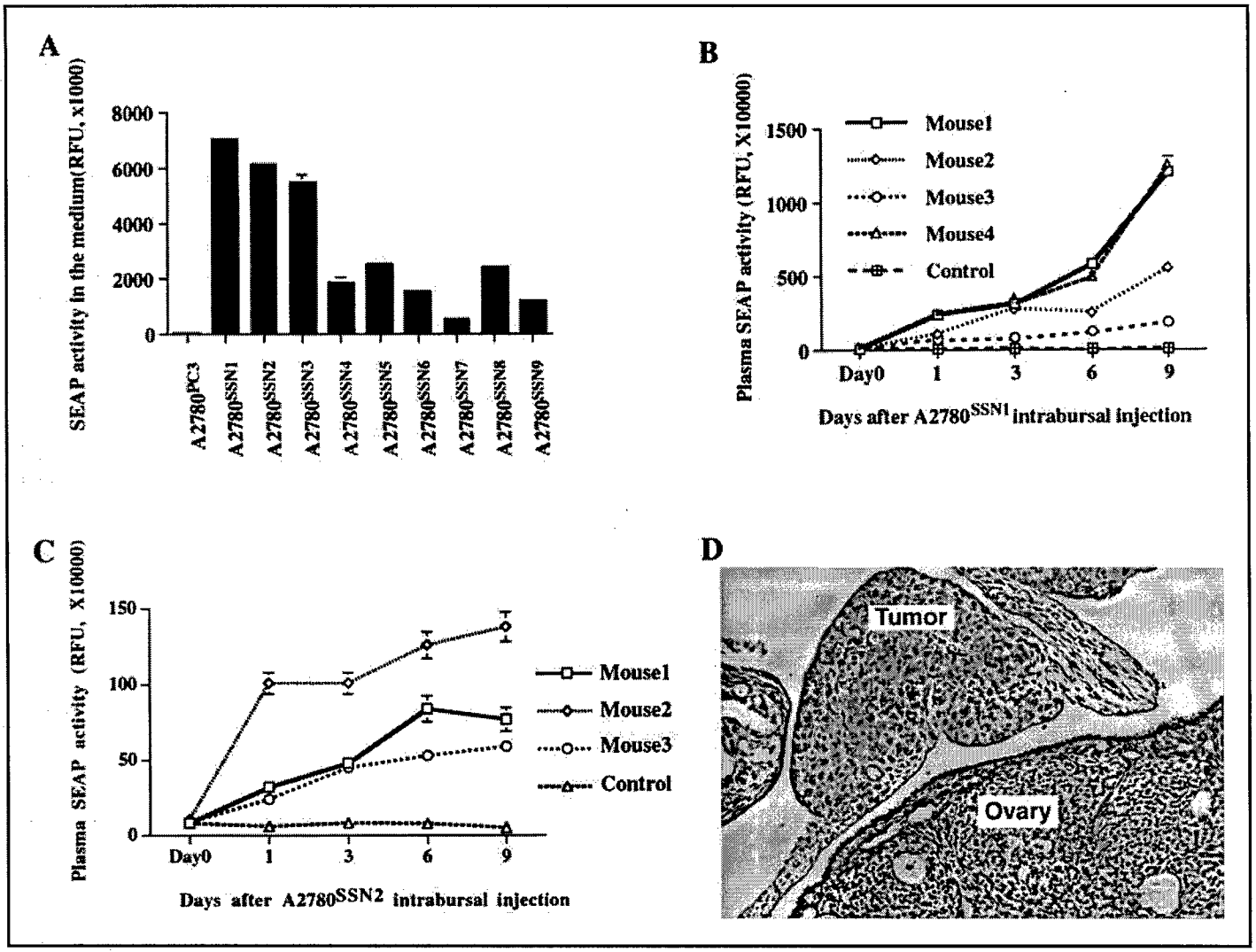

Fig. 4. Activity of the survivin promoter in stable A2780 transfectants. **A)** *In vitro* activity of survivin promoter in nine stable transfectants of A2780 ($A2780^{SSN1-9}$), assessed as secreted alkaline phosphatase (SEAP) activity, compared with that in A2780 transfected with promoterless vector ($A2780^{PC3}$). RFU = relative fluorescence units. **B)** *In vivo* activity of survivin promoter after 0.8×10^6 $A2780^{SSN1}$ cells were injected into the ovarian intrabursal space of severe combined immunodeficient (SCID) mice. The control animal was injected with 0.8×10^6 A2780 cells stably transfected with promoterless vector ($A2780^{PC3}$). **C)** *In vivo* activity of the survivin promoter after another stable transfectant $A2780^{SSN2}$ was injected into the ovarian intrabursal space of SCID mice. **D)** Section of mouse ovary stained with hematoxylin–eosin. The mouse was injected with 0.8×10^6 $A2780^{SSN1}$ cells and killed 14 days after tumor implantation. Data are the mean of three determinations. **Error bars** are 95% confidence intervals.

of the mouse and human genes is high *(6)*. The discrepancy, however, could be related to age. Our initial analysis used young adult mice before the onset of thymic atrophy, as did the earlier report *(18)*. Consequently, we investigated whether an age-related change in survivin expression occurred in the mouse thymus and found that the level of survivin protein was markedly lower in older adult mice than in younger adult mice.

A SAGE (serial analysis of gene expression) analysis found that survivin transcripts were the fourth most frequently overexpressed transcript in common human cancers (e.g., melanoma and cancers of the colon, brain, breast, and lung) relative to levels in normal cells *(5)*, suggesting that survivin was a potential target for cancer therapy. If increased survivin activity is controlled transcriptionally, then the survivin promoter might control transgene expression in a cancer-specific manner. Transcriptional regulation of survivin expression in cancer cells has indeed been reported *(6)*. Using approximately 1 kilobase of the 5′ upstream regulatory region of the survivin gene to drive SEAP expression in ovarian, mammary, colon, lung, and uterine cervical cancer cell lines, we have shown that the survivin promoter can control gene expression regardless of tumor type, mechanism of oncogenesis, and species, and we have confirmed that survivin expression appears to be, at least in part, transcriptionally activated.

In contrast to adult tissues, where survivin expression is largely limited to activation during oncogenesis, in the human fetus, survivin is abundantly expressed in apoptosis-regulated tissues. Similarly, survivin was nearly ubiquitously expressed in embryonic mouse tissues at an early gestational stage (embryonic day 11.5) but was later expressed more selectively *(16)*. Increased survivin expression and survivin promoter activity in cancer cell lines indicate that transcriptional factors needed for survivin transcription reappear or are reactivated during oncogenesis. The approximately 1-kilobase fragment of the survivin promoter used in this study overlaps with the 5′ portion of the gene studied by Li and Altieri *(6)* and contains the CHR (cell

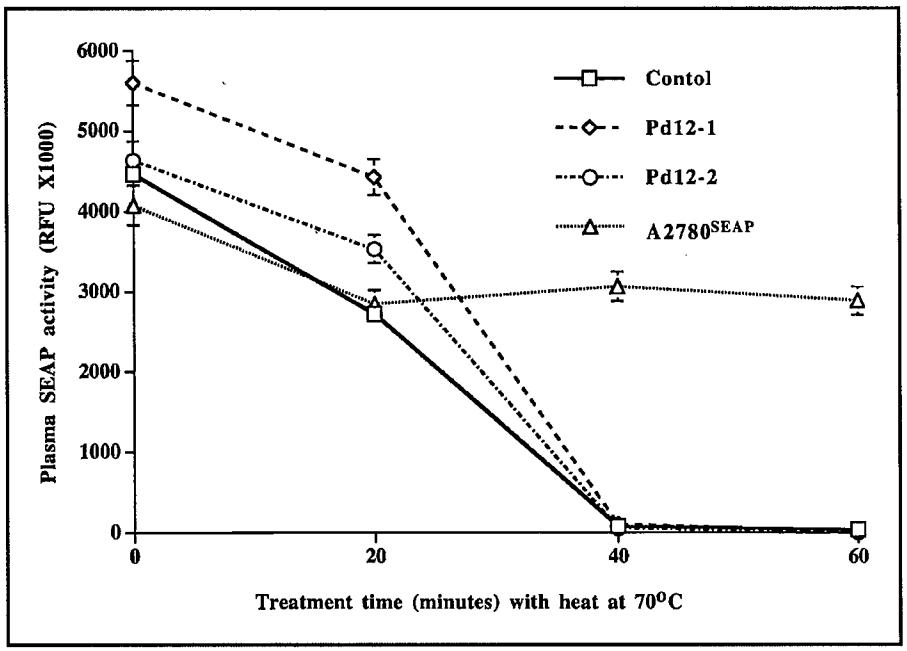

Fig. 5. Heat sensitivity of secreted alkaline phosphatase (SEAP) compared with that of endogenous alkaline phosphatase. Two microliters of plasma was used for each determination except for $A2780^{SEAP}$, where the plasma was diluted 1 : 100. For the control curve, plasma was obtained from a normal C57BL/6 mouse. For the curves Pd12–1 and Pd12–2, plasma was obtained from two C57BL/6 pregnant mice carrying fetuses of age embryonic day 12. For the $A2780^{SEAP}$ curve, plasma was obtained from a CB17/ICR severe combined immunodeficient mouse carrying a $A2780^{SEAP13}$ tumor. Data are the mean of three determinations. **Error bars** are 95% confidence intervals.

cycle gene homology region) and abundant SP_1 and CDE *(6)* as well as E_2F *(12)* transcription factor binding sites that they believe are responsible for controlling the transcription of survivin.

As indicated above, our interest in the survivin promoter first arose because of a desire to drive transgene expression in a cancer-specific manner for cancer gene therapy, to improve gene delivery progress, to specifically regulate expression of transgenes to limit the toxicity of therapeutic genes such as herpes simplex virus thymidine kinase, and to create a tumor-selective replicative oncolytic virus. We believe that the survivin promoter's specificity and expression in many early-stage cancers make it an excellent candidate for these purposes *(17,19)*. Finally, we believe that this cancer-specific reporter gene system could have major implications for monitoring tumor initiation and progression in tumor-prone transgenic animals.

References

(1) Ambrosini G, Adida C, Altieri DC. A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma. Nat Med 1997;3:917–21.

(2) Ambrosini G, Adida C, Sirugo G, Altieri DC. Induction of apoptosis and inhibition of cell proliferation by survivin gene targeting. J Biol Chem 1998;273:11177–82.

(3) Uren AG, Pakusch M, Hawkins CJ, Puls KL, Vaux DL. Cloning and expression of apoptosis inhibitory protein homologs that function to inhibit apoptosis and/or bind tumor necrosis factor receptor-associated factors. Proc Natl Acad Sci U S A 1996;93:4974–8.

(4) LaCasse EC, Baird S, Korneluk RG, MacKenzie AE. The inhibitors of apoptosis (IAPs) and their emerging role in cancer. Oncogene 1998;17: 3247–59.

(5) Velculescu VE, Madden SL, Zhang L, Lash AE, Yu J, Rago C, et al. Analysis of human transcriptomes. Nat Genet 1999;23:387–8.

(6) Li F, Altieri DC. Transcriptional analysis of human survivin gene expression. Biochem J 1999;344:305–11.

(7) Testa J, Getts L, Salazar H, Liu Z, Handel L, Godwin A, et al. Spontaneous transformation of rat ovarian surface epithelial cells results in well to poorly differentiated tumors with a parallel range of cytogenetic complexity. Cancer Res 1994;54:2778–84.

(8) Roby KF, Taylor CC, Sweetwood JP, Cheng Y, Pace JL, Tawfik O, et al. Development of a syngeneic mouse model for events related to ovarian cancer. Carcinogenesis 2000;21:585–91.

(9) Jorcyk CL, Liu ML, Shibata MA, Maroulakou IG, Komschlies KL, McPhaul MJ, et al. Development and characterization of a mouse prostate adenocarcinoma cell line: ductal formation determined by extracellular matrix. Prostate 1998;34:10–22.

(10) Shibata MA, Jorcyk CL, Liu ML, Yoshidome K, Gold LG, Green JE. The C3(1)/SV40 T antigen transgenic mouse model of prostate and mammary cancer. Toxicol Pathol 1998;26:177–82.

(11) Auersperg N, Maines-Bandiera S, Booth J, Lynch H, Godwin A, Hamilton T. Expression of two mucin antigens in cultured human ovarium surface epithelium: influence of a family history of ovarian cancer. Am J Obstet Gynecol 1995;173:558–65.

(12) Hoffmann WH, Biade S, Zilfou JT, Chen, J, Murphy M. Transcriptional repression of the anti-apoptotic survivin gene by wild type p53. J Biol Chem 2002;277:3247–57.

(13) Selvakumaran M, Bao R, Crijns AP, Connolly DC, Weinstein JK, Hamilton TC. Ovarian epithelial cell lineage-specific gene expression using the promoter of a retrovirus-like element. Cancer Res 2001;61:1291–5.

(14) Bao R, Selvakumaran M, Hamilton TC. Use of a surrogate marker (human secreted alkaline phosphatase) to monitor *in vivo* tumor growth and anticancer drug efficacy in ovarian cancer xenografts. Gynecol Oncol 2000; 78:373–9.

(15) Bronstein I, Fortin JJ, Voyta JC, Juo RR, Edwards B, Olesen CE, et al. Chemiluminescent reporter gene assays: sensitive detection of the GUS and SEAP gene products. Biotechniques 1994;17:172–4, 76–7.

(16) Adida C, Crotty PL, McGrath J, Berrebi D, Diebold J, Altieri DC. Developmentally regulated expression of the novel cancer anti-apoptosis gene survivin in human and mouse differentiation. Am J Pathol 1998;152:43–9.

(17) Lu CD, Altieri DC, Tanigawa N. Expression of a novel anti-apoptosis gene, survivin, correlated with tumor cell apoptosis and p53 accumulation in gastric carcinomas. Cancer Res 1998;58:1808–12.

(18) Kobayashi K, Hatano M, Otaki M, Ogasawara T, Tokuhisa T. Expression of a murine homologue of the inhibitor of apoptosis protein is related to cell proliferation. Proc Natl Acad Sci U S A 1999;96:1457–62.

(19) Tanaka K, Iwamoto S, Gon G, Nohara T, Iwamoto M, Tanigawa N. Expression of survivin and its relationship to loss of apoptosis in breast carcinomas. Clin Cancer Res 2000;6:127–34.

Notes

Supported in part by grants CA06927, CA51228, CA56916, and CA84242 from the National Institutes of Health, Specialized Program of Research Excellence (SPORE) grant CA83638, an appropriation from the Commonwealth of Pennsylvania, the Adler Foundation, Edgar Astrove, and the Evy Lessin Fund (T. C. Hamilton).

We thank Dr. Randy Hardy for helpful discussion with regard to the development and involution of the mouse thymus. We also thank Dr. Paul Terranova for providing the transformed IG10, IF5, and MOSE cell lines and helpful discussion with regard to isolation and culture of primary MOSE cells.

Manuscript received September 28, 2001; revised January 25, 2002; accepted February 4, 2002.

nature
cell biology

Mitochondrial p53 activates Bak and causes disruption of a Bak–Mcl1 complex

J. I-Ju Leu[1], Patrick Dumont[2], Michael Hafey[2], Maureen E. Murphy[2] and Donna L. George[1]

The tumour suppressor activity of the p53 protein has been explained by its ability to induce apoptosis in response to a variety of cellular stresses[1,2]. Thus, understanding the mechanism by which p53 functions in the execution of cell death pathways is of considerable importance in cancer biology. Recent studies have indicated that p53 has a direct signalling role at mitochondria in the induction of apoptosis[3–6], although the mechanisms involved are not completely understood. Here we show that, after cell stress, p53 interacts with the pro-apoptotic mitochondrial membrane protein Bak. Interaction of p53 with Bak causes oligomerization of Bak and release of cytochrome *c* from mitochondria. Notably, we show that formation of the p53–Bak complex coincides with loss of an interaction between Bak and the anti-apoptotic Bcl2-family member Mcl1. These results are consistent with a model in which p53 and Mcl1 have opposing effects on mitochondrial apoptosis by interacting with, and modulating the activity of, the death effector Bak.

The role of p53 in apoptosis is multi-faceted and most probably involves its transcriptional regulatory functions, as well as less well-characterized transcription-independent activities[1,2]. Recent studies have demonstrated that a fraction of stress-activated p53 translocates to mitochondria after an apoptotic stimulus, but not during p53-dependent growth arrest[3,4]. Further support for the significance of p53 mitochondrial localization followed from our analysis of the apoptotic potential of two polymorphic variants of p53 (ref. 5). It has been known for some time that a common coding region polymorphism occurs in human p53, resulting in either an arginine (R72) or proline (P72) at amino acid 72. The frequency of the polymorphic variants of p53 varies in different populations: for example, approximately 35–40% of Caucasians, but only 10–12% of African Americans, are homozygous for the R72 allele[7]. In an earlier study, we found that the R72 variant of p53 exhibits a greater ability to induce apoptosis than does the P72 form, and at least one reason for this difference is enhanced localization of the R72 variant to mitochondria[5]. The greater mitochondrial localization of the R72 variant correlates with enhanced nuclear export, suggesting that differences in nucleo-cytoplasmic shuttling may influence trafficking of p53 to mitochondria.

Reasoning that p53 functions with one or more mitochondrial factors to execute its pro-apoptotic role, we sought to identify p53-bound mitochondrial proteins. As a source of mitochondrial p53, we used an inducible Saos2 cell line that stably expresses a temperature-sensitive (ts)-R72 variant of p53 (ref 5). The ts-p53 protein exists in a denatured, inactive, form at 39 °C and becomes wild type in conformation and activity at 32 °C. Importantly, a significant fraction of the p53 protein localizes to mitochondria after an apoptosis-inducing temperature shift to 32 °C (see ref. 5 and Fig. 1a). Among several protein bands that were detectable in mitochondrial p53 immunoprecipitates from cells incubated at 32 °C, but not at 39 °C, was a major species with a relative molecular mass (M_r) of approximately 28,000 (Fig. 1a). This band was excised from SDS–PAGE gels, subjected to tryptic digestion, and the resulting peptides were analysed by liquid chromatography and tandem mass spectrometry. Two tryptic fragments of the protein Bak were identified (Fig. 1a). Bak is a multidomain pro-apoptotic member of the Bcl-2 family of proteins, which are key regulators of programmed cell death[8]. We then verified that Bak co-immunoprecipitates with p53 from mitochondrial lysates using immunoprecipitation-western blot analysis (Fig. 1a).

Bak is an integral protein of the mitochondrial membrane, and the R72 form of p53 localizes to mitochondria more effectively than the P72 variant. Therefore, we addressed whether these two p53 isoforms would exhibit differences in binding to Bak *in vivo* that would correlate with apoptotic potential. Analysis of whole-cell and mitochondrial lysates by immunoprecipitation-western blotting confirmed that, compared with P72, much more of the R72 variant is complexed with Bak (Fig. 1b, c). A longer exposure of the autoradiogram detected the less abundant interaction of P72 with Bak (data not shown). In these analyses, little association was detected between p53 and two other Bcl-2 family members, the pro-apoptotic protein Bax and the anti-apoptotic protein Bcl-x_L, both of which were reported to function in mitochondrial apoptosis induction by p53 (refs 4, 6). Similarly, *in vitro* binding assays using *in-vitro*-translated proteins identified a robust association of p53 with Bak, but failed to identify a detectable interaction of p53 with either Bax or Bcl-x_L (Fig. 1d). We extended these studies to verify that the interaction between p53 and Bak occurs in different cell lines expressing endogenous wild-type p53. The human prostate carcinoma cell line LNCaP and the human breast carcinoma

[1]Department of Genetics, University of Pennsylvania School of Medicine, 422 Curie Boulevard, Philadelphia, PA 19104, USA. [2]Department of Pharmacology, Fox Chase Cancer Center, 333 Cottman Avenue, Philadelphia, PA 19111, USA.
Correspondence should be addressed to D.L.G. (e-mail: georged@mail.med.upenn.edu)

Published online: 11 April 2004; DOI: 10.1038/ncb1123

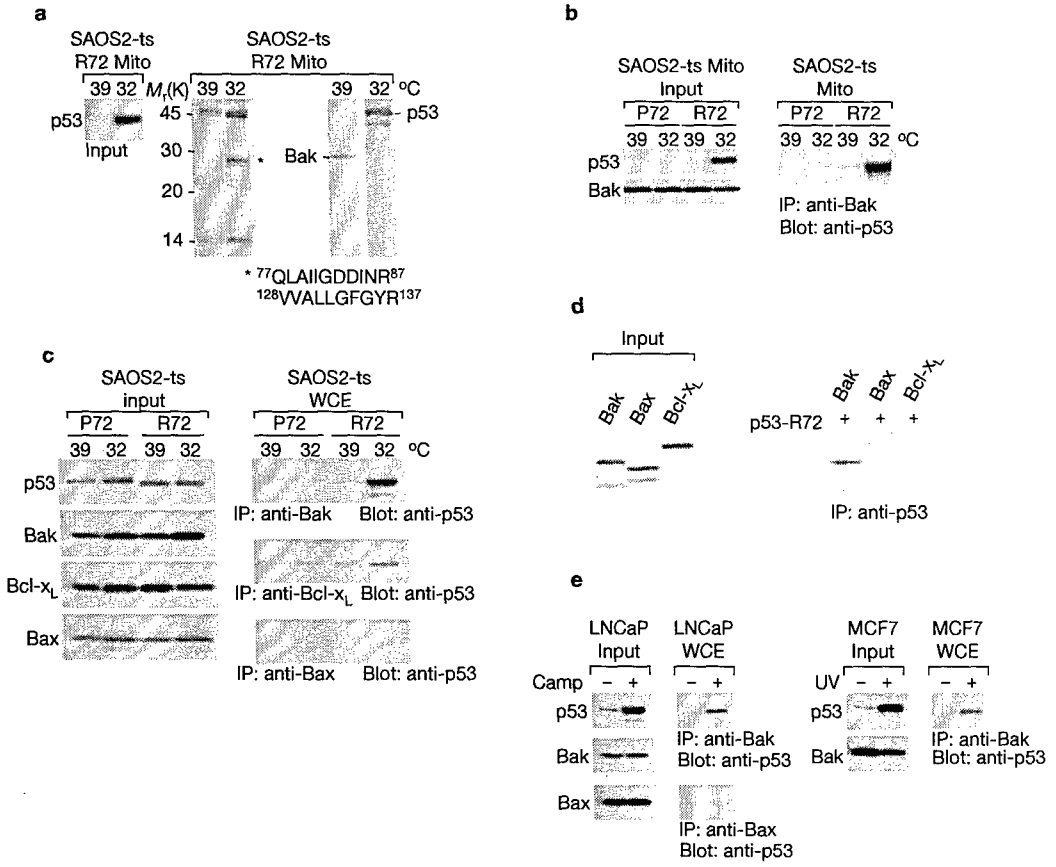

Figure 1 Bak and p53 interact. (**a**) Immunoblotting of mitochondrial p53 in ts-p53(R72)-Saos2 cells grown at 39 °C or 32 °C (left). Mitochondrial extracts were immunoprecipitated using agarose-conjugated anti-p53 (middle). The silver-stained protein band of approximately 28K was excised and subjected to tryptic digestion and liquid chromatography-tandem mass spectrometry. Asterisk indicates the peptide sequences of Bak obtained from this analysis. Immunoblots of proteins co-precipitating with p53 from cells at 32 °C were probed with antibodies to Bak and p53, as indicated (right). (**b**) Immunoblots of mitochondrial extracts from ts-P72-Saos2 and ts-R72-Saos2 cells were analysed with an antibody against p53 or Bak (left). The same extracts were immunoprecipitated (IP) with anti-Bak followed by immunoblotting for associated p53 (right). (**c**) Immunoblots of whole-cell extracts (WCEs) from ts-P72-Saos2 and ts-R72-Saos2 cells analysed for p53, Bak, Bcl-x_L and Bax (left). IP of the same lysates for Bak, Bcl-x_L or Bax, followed by immunoblot analysis for p53 (right). The autoradiogram exposure times were equivalent for the Bak, Bcl-x_L and Bax IP blots, and the results depicted were consistent with different antibodies for each protein. (**d**) *In-vitro*-translated full-length p53 (R72) was mixed with *in-vitro*-translated-^{35}S-labelled full-length Bak, Bax or Bcl-x_L. Input analyses confirm similar expression levels for the proteins. Binding to p53 was assayed by IP with an antibody to p53. The products were analysed by SDS–PAGE and autoradiography (right). (**e**) Immunoblots of whole-cell extracts from LNCaP cells (± 5 μM camptothecin for 24 h) or MCF7 cells (± 7.5 J m^{-2} ultraviolet irradiation) were probed with anti-p53, anti-Bak or anti-Bax, as indicated. IP of the same lysates with anti-Bak or anti-Bax, followed by blotting with anti-p53 (right). For these and similar assays, protein interactions were examined after solubilization of cellular fractions in Chaps, which, unlike non-ionic detergents, does not induce an activating, conformational change in Bak or Bax[12,30].

cell line MCF7 were treated with camptothecin or ultraviolet irradiation, respectively, to induce upregulation of p53. Immunoprecipitation-western blot analysis confirmed that after its stress-induced activation, p53 interacts with Bak (Fig. 1e). In these cells, as with the p53-Saos2 transfectants, a much stronger association was detected between p53 and Bak, than between p53 and Bax (Fig. 1e) or Bcl-x_L (data not shown).

To map the domain of p53 that interacts with Bak, we used glutathione *S*-transferase (GST) pull-down assays, in which ^{35}S-labelled *in-vitro*-translated Bak (full-length) bound to immobilized GST–p53 (full-length), but not to GST alone (Fig. 2a). In these *in vitro* assays, the interaction of the R72 and P72 forms of p53 with Bak was indistinguishable (Fig. 2a); this is consistent with our conclusion that the greater association of R72 to Bak observed *in vivo* (Fig. 1b) reflects the enhanced nuclear export and mitochondrial localization of this variant in cells[5]. Bak does not bind to the amino-terminal domain (amino acids 1–92), or to the carboxy-terminal domain (amino acids 318–393), of p53. Instead, the central region of p53, encompassing amino acids 92–318, contains the Bak-binding domain (Fig. 2b). In fact, these data suggest that there may be two Bak-interacting domains within the central DNA-binding domain of p53. Other proteins, including mSin3a and TBP, also have been found to interact with two distinct regions of the p53 protein[9,10]. We used similar assays to map the p53-interacting domain of Bak. These analyses demonstrated that the introduction of mutations within critical residues of any of the three Bcl-2-homology regions of Bak (BH1, BH2, BH3) resulted in substantially reduced p53 binding (Fig. 2c). Existing data suggest that these three segments of Bak most probably form a binding pocket that would be disrupted by mutations within any one of the domains[11,12]. Thus, *in vitro* binding analysis suggests that p53

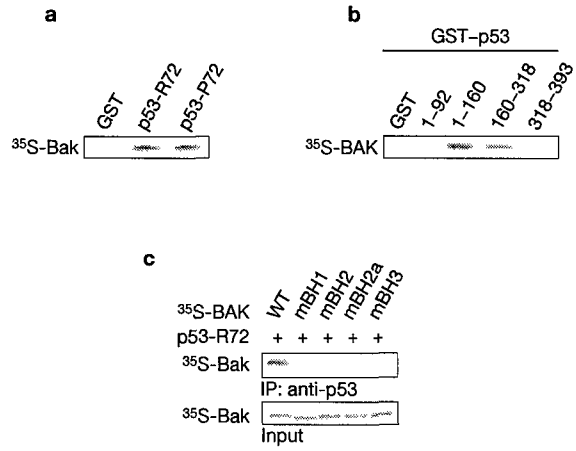

Figure 2 p53 binds to Bak *in vitro*. **(a)** GST pull-down assays were performed using *in-vitro*-translated-^{35}S-labelled full-length human Bak and GST-tagged recombinant proteins: GST alone, GST–p53-R72 or GST–p53-P72. Protein complexes were analysed by SDS–PAGE and fluorography. **(b)** *In-vitro*-translated ^{35}S-labelled full-length human Bak was mixed with GST alone, or GST-tagged proteins containing different amino-acid residues of p53, as indicated. **(c)** *In-vitro*-translated full-length p53-R72 was mixed with *in-vitro*-translated ^{35}S-labelled full-length wild-type Bak, or the different BH-domain Bak mutants indicated (see Methods for the amino-acid changes in the Bak mutants). p53–Bak interactions were assayed by IP with anti-p53. The products were resolved by 10–20% SDS–PAGE and the autoradiogram was developed.

interacts directly with Bak through a pocket structure formed by the BH1, BH2 and BH3 domains.

In unstressed cells, Bak is located at the mitochondrial outer membrane as an inactive monomer. After diverse apoptotic stimuli, Bak undergoes an activating conformational change that results in the formation of higher-order multimers; this leads to the release of cytochrome *c* and other pro-apoptogenic factors from mitochondria into the cytosol[13–15]. To assess the ability of p53 to induce oligomerization of Bak, we used a well-established assay using highly purified mitochondria isolated from sucrose gradients[14,15]. The addition of full-length recombinant p53 (R72 or P72) to mitochondria isolated from unstressed p53-null cells readily shifted Bak from a monomer into higher-order multimers (Fig. 3a). Using deletion mutants of p53, we confirmed that the ability to bind to Bak correlates positively with their ability to induce Bak oligomerization, as well as to stimulate release of cytochrome *c* from purified mitochondria (compare Figs 2b and 3a). Activation of Bak is accompanied by conformational changes, resulting in the exposure of an amino-terminal epitope of Bak that becomes susceptible to trypsin digestion[14,16,17]. Therefore, we also examined whether p53 could induce such a conformational change in Bak. First, we confirmed that Bak exists in a relatively inactive trypsin-resistant form in untreated mitochondria (Fig. 3b). Significantly, incubation of mitochondria with recombinant p53, but not GST alone, resulted in oligomerization of Bak and an increased susceptibility of Bak to trypsin digestion. This enhanced susceptibility to trypsin proteolysis was consistent with two different antibodies directed against the Bak N terminus (Fig. 3b). Together, these data demonstrate that, similarly to other molecules that activate and oligomerize Bak, p53 induces an amino-terminal conformational change in Bak that is associated with Bak oligomerization. To determine whether p53-induced cytochrome *c* release is dependent on the ability of p53 to bind and oligomerize Bak, we isolated mitochondria from mouse embryo fibroblasts (MEFs) derived from wild type (*Bak*$^{+/+}$) and Bak-deficient (*Bak*$^{-/-}$) mice[18]. Consistent with our results obtained from mitochondria purified from cultured human cells, p53 readily induced cytochrome *c* release from mitochondria isolated from wild-type MEFs. In contrast, incubation with p53 failed to release cytochrome *c* from the Bak-deficient mitochondria (Fig. 3c). These data further support a model in which p53, operating at the mitochondria, binds directly to Bak, resulting in Bak oligomerization and cytochrome *c* release.

To better understand the role of mitochondrial p53 in apoptosis, we focused on identifying mitochondrial signalling pathways that might be altered, or mediated, by the p53–Bak interaction. It has been proposed that in unstressed cells, Bak is complexed with other mitochondrial proteins that function to maintain this pro-apoptotic factor in an inactive conformation[12,16]. Anti-apoptotic members of the Bcl-2 family are attractive candidates to perform such a role. In unstressed cells, however, the majority of the anti-apoptotic Bcl-2 and Bcl-x_L are in different cellular compartments and do not seem to interact appreciably with inactive Bak (refs 12, 19, 20). The anti-apoptotic Mcl1 protein, however, is predominantly mitochondrial in healthy cells (ref. 21 and see Fig. 4a), and has been shown to bind to Bak and Bax in yeast two-hybrid analyses, but not to Bcl-x_L or Bcl-2 (ref. 22). To confirm that mammalian Mcl1 is complexed with Bak in unstressed cells, we performed immunoprecipitation-western blot analyses in unstressed Saos2 cells. These analyses identified an interaction of mitochondrial Mcl1 with Bak; in contrast, very little interaction of Mcl1 with Bax or Bcl-x_L was detected in these assays (Fig. 4b). A recent report has demonstrated that Bak is, in fact, complexed with Mcl1 in healthy cells[16]. We performed *in vitro* mapping experiments of this interaction and determined that an intact BH3 domain of Bak is necessary for an efficient interaction with Mcl1 (Fig. 4c).

Elevated Mcl1 expression is associated with increased protection from cell death[20,23–25]. We found that in isogenic cell lines with different levels of Mcl1, increased Mcl1 attenuates Bak oligomerization (Fig. 4d). These data suggest that Mcl1 and p53 have opposing roles in modulating Bak function. Thus, we sought to determine whether the stress-induced activation of p53 alters the Bak–Mcl1 interaction. For these analyses, we took advantage of two well-established cell lines that are isogenic, except for their p53 status. The ovarian teratocarcinoma cell line PA1 contains wild-type p53. However, its derivative PA1-E6 expresses the human papillomavirus 16 E6 protein, which targets p53 for degradation. It was shown previously[20,21] that Mcl1 is a short-lived protein, and that agents such as ultraviolet irradiation and etoposide (which can inhibit new protein synthesis) reduced cellular Mcl1 levels[20,24]. As this would interfere with our analyses, we sought stress-induction protocols that would not significantly decrease RNA or protein synthesis and Mcl1 levels. Treatment of cells with the DNA-damaging agent doxorubicin did not result in decreased steady-state levels of Mcl1, as illustrated for the PA1 and PA1-E6 cells (Fig. 5a). Notably, induction of p53 by doxorubicin resulted in a significant decrease in the Bak–Mcl1 interaction. In PA1-E6 cells lacking p53, however, the amount of Mcl1 co-precipitating with Bak was not altered by doxorubicin treatment (Fig. 5a). Similarly, there was no decrease in cellular Mcl1 protein abundance in p53-inducible Saos2 cells after temperature shift and apoptosis induction at 32 °C; however, p53 induction was accompanied by a substantial decrease in the amount of Mcl1 complexed to Bak (Fig. 5b). Thus, p53 activation results, directly or indirectly, in disruption of the Bak–Mcl1 complex.

The stress-induced localization of p53 to mitochondria is rapid and precedes mitochondrial alterations, such as caspase activation and cytochrome *c* release[3,4]. Therefore, we assessed whether the interaction of p53 and Bak correlates temporally with disruption of the Bak–Mcl1

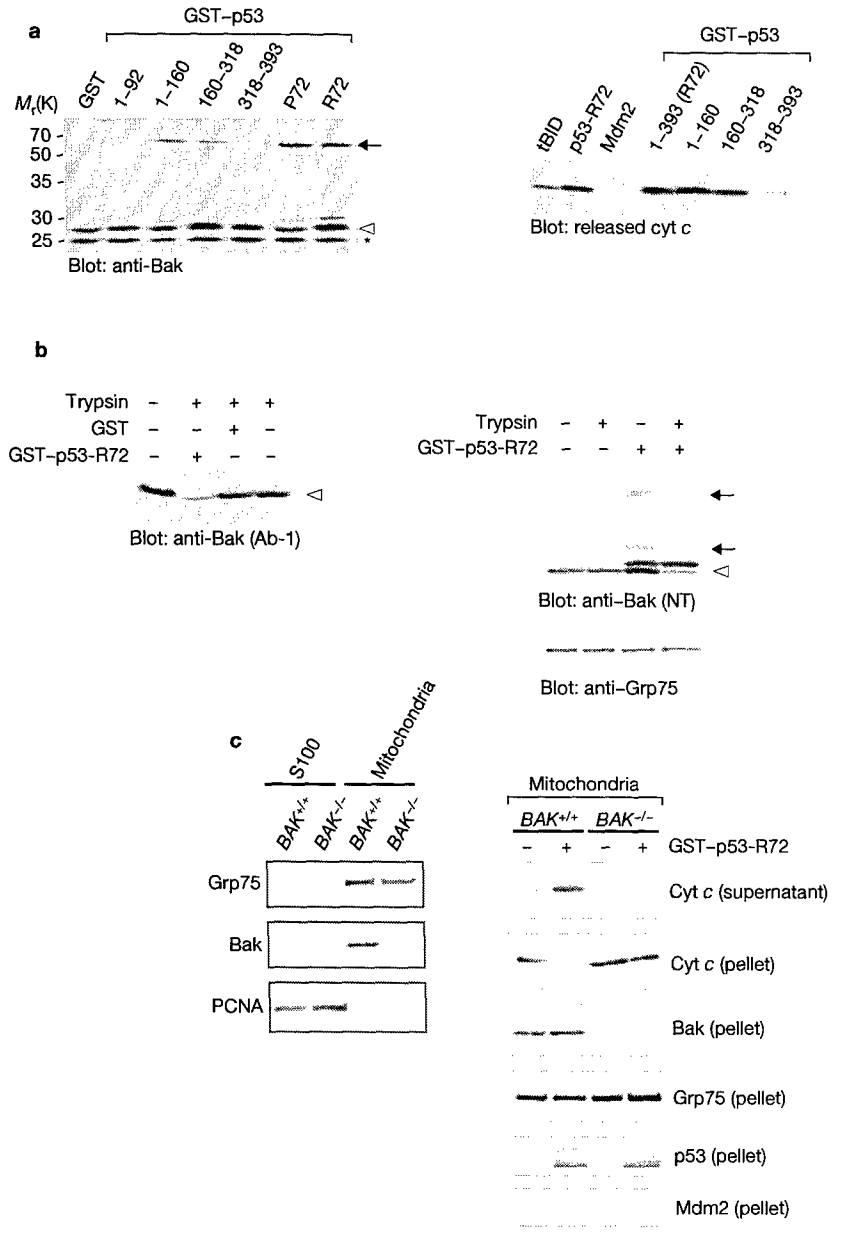

Figure 3 p53 induces oligomerization of Bak. (**a**) Mitochondria (20 μg) were incubated with 30 pmol GST–p53-R72 proteins and then crosslinked with BMH. Bak homo-oligomers were detected by immunoblotting with anti-Bak NT (left). Black arrowhead indicates Bak multimers; open arrowhead indicates Bak monomers; asterisk indicates an intramolecular crosslinked species of Bak that is sometimes detected after BMH treatment[14]. In the right panel, mitochondria were incubated with 60–100 pmol of the GST–p53-R72 proteins indicated, GST–tBid (positive control for cytochrome *c* release) or GST–Mdm2 (negative control). The supernatant fraction was analysed for released cytochrome *c* by immunoblotting. (**b**) Mitochondria were incubated in the presence or absence of 25 pmol GST–p53, GST alone, or no protein, with or without trypsin treatment. Samples were then immunoblotted with antibody raised against the Bak N terminus (anti-Bak Ab-1; left). Mitochondria were incubated in the presence or absence of 125 pmol GST–p53-R72, with or without trypsin treatment, and immunoblotted with antibody raised against the Bak N terminus (anti-Bak NT; right). The difference in degree of Bak oligomerization detected in the left versus right panels depends on the amount of GST–p53 protein used. Under the same conditions, the mitochondrial matrix protein Grp75 is trypsin-resistant. (**c**) Cytosolic S100 and mitochondrial fractions from *Bak*$^{+/+}$ and *Bak*$^{-/-}$ MEFs were immunoblotted for mitochondrial proteins, GRP75 and Bak, and the nuclear-cytoplasmic protein PCNA, demonstrating the integrity of the mitochondrial preparations and absence of contamination from lysed nuclei (left). Mitochondria from *Bak*$^{+/+}$ and *Bak*$^{-/-}$ MEFs were incubated in the presence or absence of GST–p53-R72 (right). Immunoblotting identifies release of cytochrome *c* from the pellet fraction to the supernatant in control, but not Bak-deficient, mitochondria. As controls, mitochondrial pellets were immunoblotted for mitochondrial proteins Bak and Grp75, added recombinant p53, and the nuclear marker Mdm2.

complex. Saos2 cells with inducible p53 were shifted from 39 °C to 32 °C to activate p53 and to induce apoptosis. At 6 h and 15 h after temperature shift, cell lysates were immunoprecipitated with an antibody to Bak before western blotting for associated p53 or Mcl1 (Fig. 5c). The results show that by 6 h, a p53–Bak complex is present, while the Bak–Mcl1 complex is no longer detectable despite the fact that mitochondrial Mcl1 levels remain unchanged (Fig. 5c). Notably, loss of the Bak–Mcl1 complex precedes caspase activation, as caspase cleavage of target proteins

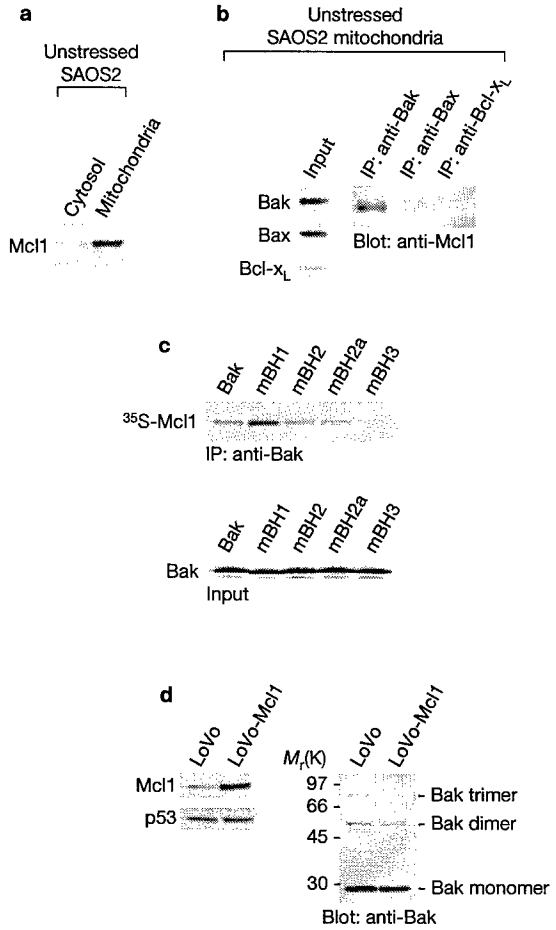

Figure 4 Characterization of the Mcl1–Bak interaction. (**a**) The presence of Mcl1 in cytosolic and mitochondrial fractions of unstressed Saos2 cells was analysed by immunoblotting with an anti-Mcl1 antibody. (**b**) To evaluate the interaction of Mcl1 with Bak, Bax and Bcl-x_L, mitochondria (300 μg) from unstressed p53-null Saos2 cells were solubilized in 1% Chaps. Mitochondrial proteins were immunoprecipitated with anti-Bak, anti-Bax or anti-Bcl-x_L antibodies, followed by immunoblotting with an anti-Mcl1 antibody. (**c**) To determine the region of Bak required for Mcl1 interaction, *in-vitro*-translated ^{35}S-labelled full-length Mcl1 was mixed with *in-vitro*-translated full-length wild-type Bak or the BH-domain Bak mutants indicated. Protein interactions were assayed by immunoprecipitation with an anti-Bak antibody before SDS–PAGE. (**d**) Overexpression of Mcl1 is associated with attenuated Bak oligomerization *in vivo*, as illustrated for LoVo colon carcinoma cells and for an Mcl1-overexpressing derivative (LoVo-Mcl1). Bak multimer formation was detected in a small percentage of cells undergoing spontaneous apoptosis in an otherwise asynchronous population. Protein expression levels for Mcl1, p53 and Bak were examined by immunoblotting.

such as Mdm2, caspase-3 and caspase-8, was not detectable at 6 h, but was clearly present by 15 h (see Fig. 5c and ref. 5). Additionally, we found that the p53–Bak complex formed independently of caspase activation, as p53–Bak complexes were detectable even in the presence of the broad-spectrum caspase inhibitor zVAD-fmk (Fig. 5d). These data reinforce the conclusion that the formation of a p53–Bak complex and the disruption of the Bak–Mcl1 interaction represent opposing upstream events in the mitochondrial cell death pathway.

Finally, to determine if p53 can disrupt the Bak–Mcl1 complex, we performed *in vitro* assays with highly purified mitochondria and recombinant p53 protein. In these studies, incubation of mitochondria with bacterially expressed His-tagged p53 protein resulted in a clear decrease in the amount of Mcl1 interacting with Bak, as tested by immunoprecipitation with an antibody to Bak and western blotting for Mcl1 (Fig. 5e). Notably, disruption of the Mcl1–Bak interaction correlated with formation of a p53–Bak complex (Fig. 5e), Bak oligomerization (data not shown) and the release of cytochrome *c* from mitochondria (Fig. 5e). In contrast, incubation of mitochondria with a control His-tagged protein, or with recombinant tBid, had no effect on the Mcl1–Bak complex (Fig. 5e). Identical results were obtained using mitochondria purified from other cells, as well as a different form of recombinant p53 (GST–p53; data not shown).

In this study, we provide new insight into the mitochondrial apoptosis pathway by demonstrating that p53 and Mcl1 have opposing actions in regulating the death effector Bak. These studies further define a non-transcriptional role for mitochondrial p53 as a direct upstream activator of Bak-mediated mitochondrial dysfunction. Our data also support a model in which the anti-apoptotic Mcl1 protein functions to maintain Bak in an inactive conformation in normal cells. This could explain why enhanced expression of Mcl1 observed in some tumours is associated with a reduced responsiveness to chemotherapy and a poor prognosis[20,23–25]. Previous studies have indicated that Mcl1 downregulation alone may not be sufficient to induce apoptosis[16,20]; our analyses are consistent with the idea that both a disruption of a Bak–Mcl1 complex, as well as activation of Bak, may be required. Accumulating data show that p53 can respond rapidly to adverse conditions by trafficking to mitochondria[3–5]; the ability of p53 to directly bind and activate Bak provides another mechanism (in addition to its transcriptional functions) for p53 to promote an enhanced, multi-faceted apoptotic response. Consistently, it has recently been reported that the pro-apoptotic Bcl-2 family member Bax can undergo oligomerization after treatment with cytoplasmic p53 (ref. 26), although no direct interaction between p53 and Bax could be detected[4,26]. These and other studies support a direct mitochondrial role for p53 in apoptosis induction, but the relative contribution of this activity to p53-mediated cell death remains to be assessed. Although it has been shown that a well-characterized mutant of p53 lacking transcriptional function is defective at apoptosis induction in mice[27], this mutant was shown to be defective at nuclear export, which we have shown is important for mitochondrial localization[5]. Therefore, that study does not shed light on this issue. Our finding that the P72 polymorphic variant of p53, which has greatly reduced ability to localize to mitochondria, also has significantly reduced ability to induce apoptosis[5], suggests that further assessment of the degree to which mitochondrial p53 contributes to apoptosis induction is warranted. □

METHODS

Cell lines and purification of mitochondria. H1299 lung1 adenocarcinoma and Saos2 osteosarcoma cells are p53 null. The following human cell lines contain wild-type p53: LNCaP prostate carcinoma (R72); MCF7 breast carcinoma (R72/P72); LoVo colon carcinoma (R72); PA1 ovarian teratocarcinoma (R72). MEF cell lines were established from *Bak*$^{+/+}$ and *Bak*$^{-/-}$ mice (kindly provided by T. Lindsten and C. Thompson, University of Pennsylvania, Philadelphia, PA). Mitochondria were purified using a differential centrifugation method as described[14,28]. Briefly, cells were harvested, centrifuged at 500*g* for 5 min at 4 °C, and resuspended in fractionation buffer A (10 mM Hepes-KOH at pH 7.4, 0.1 mM EDTA, 1 mM EGTA and 250 mM sucrose) supplemented with protease inhibitors (6 μg ml^{-1} aprotinin, 6 μg ml^{-1} leupeptin and 0.5 mM phenylmethylsulphonyl fluoride (PMSF)). Cell disruption was performed by passing the cells through a 23-gauge needle 3–5 times. The homogenates were spun at 700*g* for 10 min at 4 °C. The supernatants were removed and spun at 7,000*g* for 10 min at 4 °C. The mitochondrial pellets were washed with fractionation buffer B

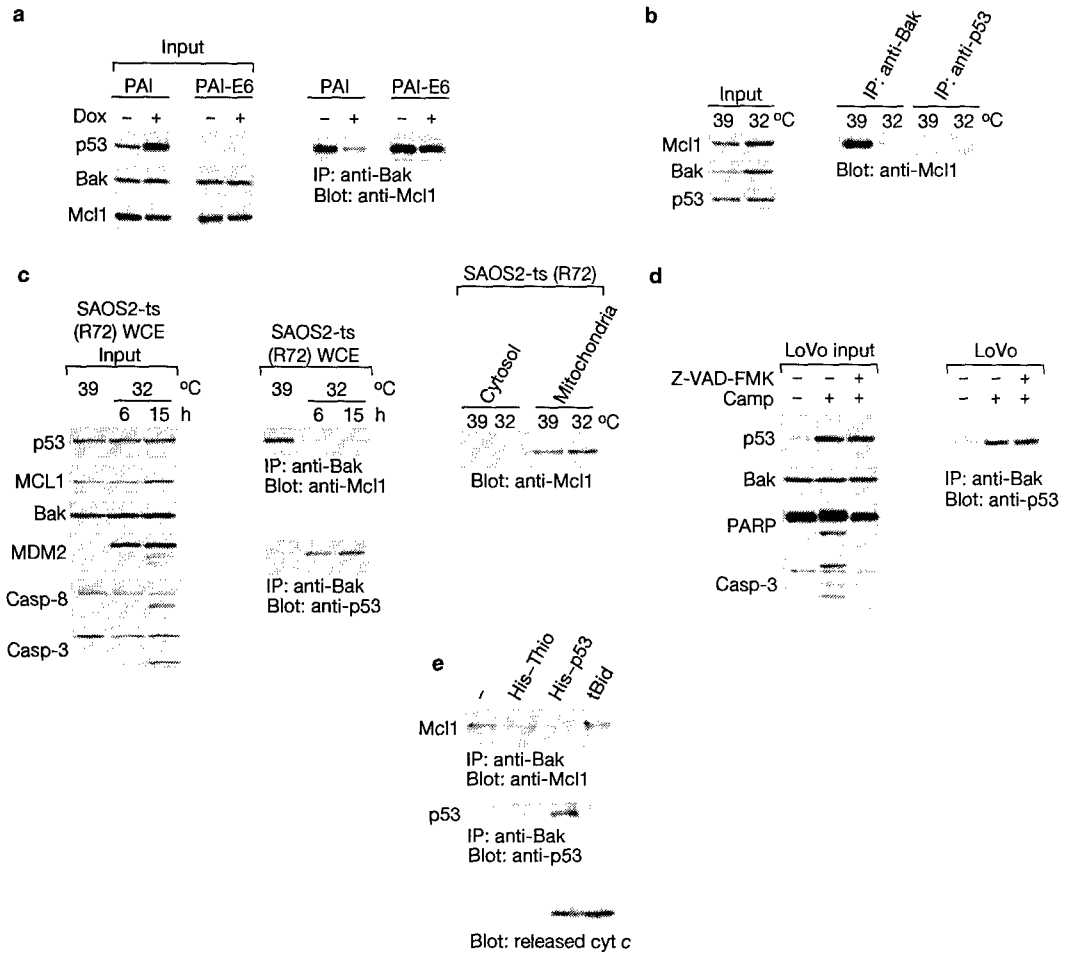

Figure 5 The p53–Bak interaction correlates with disruption of the Mcl1–Bak interaction. (**a**) Whole-cell extracts (100 μg) from PA1 and PA1-E6 cells, either left untreated or treated with doxorubicin (Dox; 0.5 μg ml^{-1}, 5 h), were analysed by immunoblotting with antibodies to Mcl1, p53 or Bak, as indicated (left). Whole-cell extracts (2 mg) from PA1 and PA1-E6 cells were immunoprecipitated with an antibody against Bak before immunoblotting for Mcl1 (right). (**b**) Whole-cell extracts from ts-R72 Saos2 cells, incubated at 39 °C or 32 °C, were analysed with antibodies raised against p53, Mcl1 or Bak, as indicated (left). The same whole-cell extracts were immunoprecipitated with anti-Bak or anti-p53 antibodies before immunoblotting for Mcl1 (right). (**c**) Whole-cell extracts from ts-R72 Saos2 cells incubated at 39 °C or 32 °C for 6 h or 15 h were analysed by immunoblotting with antibodies against p53, Mcl1, Bak, Mdm2, caspase-8 or caspase-3, as indicated (left). The same extracts were immunoprecipitated with an anti-Bak antibody before immunoblotting for Mcl1 or p53 (middle). Cytosolic and mitochondrial fractions from ts-R72 Saos2 cells incubated at 39 °C or 32 °C for 6 h were analysed by immunblotting for Mcl1 (right). (**d**) LoVo cells were treated with camptothecin ± zVAD-fmk for 5 h before immunoblotting for p53, Bak, PARP or caspase-3 (left). The same extracts were subjected to immunoprecipitation with an antibody against Bak before immunoblotting for p53 (right). (**e**) Purified mitochondria were incubated with the indicated recombinant proteins for 30 min at 30 °C. The mitochondria were pelleted, solubilized in 1% Chaps, immunoprecipitated with an antibody to Bak and analysed by immunoblotting for Mcl1 (top). The same blot was reprobed with an antibody against p53 (middle). The supernatant fraction was analysed for released cytochrome *c* by immunoblotting (bottom).

(10 mM Hepes-KOH at pH 7.4, 5 mM KH_2PO_4, 5 mM succinate and 250 mM sucrose) and resuspended in fractionation buffer B to a final protein concentration of 2–3 mg ml^{-1}. Protein concentration was determined using the Bradford Reagent (Bio-Rad Laboratories, Inc., Hercules, CA).

Identification of mitochondrial p53-interacting proteins. Mitochondria were purified from the ts-R72 Saos2 cells incubated at 39 °C or 32 °C for 22 h and lysed in 1% Chaps Buffer (5 mM $MgCl_2$, 137 mM KCl, 1 mM EDTA, 1 mM EGTA, 1% Chaps and 20 mM Tris-HCl at pH 7.5) supplemented with protease inhibitors. For immunopurification, an anti-p53 antibody conjugated to agarose resin (EMD Biosciences, Inc., Oncogene Research Products, San Diego, CA) was used. After five washes with 1% Chaps buffer, the associated proteins were resolved by SDS–PAGE in a 10–20% gradient gel (Cambrex Bio Science Rockland, Inc., Rockland, ME) and visualized by silver stain (Bio-Rad). The silver-stained band corresponding to ~28K was excised from the gel, subjected to trypsin digestion, and the resulting peptides were analysed by liquid chromatography-tandem mass spectrometry at the Genomics Institute and Abramson Cancer Center Proteomics Core Facility (University of Pennsylvania School of Medicine, Philadelphia, PA).

Recombinant protein production. Recombinant GST-tagged proteins were generated using the pGEX-4T-3 vector (Amersham Biosciences, Piscataway, NJ), and recombinant His-tagged proteins were produced using pET-32 EK/LIC vector (EMD Biosciences). The GST-tagged proteins were induced for 6 h with 0.1 mM IPTG in BL21 cells doubly deficient for glutathione reductase and thioredoxin reductase[29]. His-tagged proteins were induced for 6 h with 0.5 mM IPTG in BL21 (DE3) cells doubly deficient for glutathione reductase and thioredoxin reductase. For purification of GST–p53, the bacterial pellets were resuspended in BugBuster HT Extraction Buffer (EMD Biosciences) and applied to glutathione–Sepharose 4B (Amersham Biosciences). The

GST–p53–glutathione–Sepharose complex was washed five times with 20 column volumes of PBS lacking calcium and magnesium ions (Invitrogen, Carlsbad, CA) and eluted with 10 mM glutathione in 50 mM Tris-HCl at pH 8.0. For purification of His–p53, the bacterial pellets were resuspended in BugBuster HT Extraction Buffer (EMD Biosciences) with 5 mM Imidazole and applied to Ni-NTA resin (Qiagen Inc., Valencia, CA). The His–p53–Ni-NTA complex was washed five times with 20 column volumes of wash buffer (50 mM NaH_2PO_4, 300 mM NaCl and 10 mM imidazole at pH 8.0) and eluted with wash buffer containing 250 mM Imidazole. The eluted GST–p53 and His–p53 were dialysed against fractionation buffer B.

Bak activation and cytochrome *c* release. For cytochrome *c* release and Bak oligomerization assays[14,15] involving human H1299 or Saos2 cells, 15 μg of mitochondria were incubated with 30–60 pmol of the indicated recombinant p53 proteins at 30 °C for 30 min. The mitochondria were pelleted at 10,000*g* for 15 min at 4 °C. Mitochondria were also isolated from *Bak*$^{+/+}$ and *Bak*$^{-/-}$ MEFs. For these studies, 15 μg of mitochondria were incubated with or without 600 pmol of the GST–p53-R72 proteins at 30 °C for 45 min. The mitochondria were pelleted at 10,000*g* for 15 min at 4 °C. After incubation, the supernatant fractions were separated by SDS–PAGE and subjected to western blotting with an anti-cytochrome *c* antibody (BD Bioscience-Pharmigen, San Diego, CA). For control reactions, mitochondrial pellet fractions were separated by SDS–PAGE in 4–20% gradient gels and subjected to western blotting using antibodies directed against the following proteins: cytochrome *c* (BD Bioscience-Pharmigen), GRP75 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), PCNA (Santa Cruz Biotechnology), Mdm2 (EMD Biosciences), Bak (anti-Bak NT, Upstate USA, Inc., Charlottesville, VA), and p53 (BD Bioscience-Pharmigen).

For detection of Bak oligomers using the uncleavable protein crosslinker 1,6-bismaleimidohexane (BMH), 20 μg of mitochondria were incubated with the indicated recombinant p53 proteins (15–30 pmol) for 1 h at 4 °C. After incubation, the proteins were crosslinked with 2.8 mM BMH (Pierce) for 30 min at 25–30 °C. The mitochondria were pelleted, dissolved in SDS sample buffer, and loaded on Nupage Novex 10% Bis-Tris gels (Invitrogen). For Bak–Mcl1 disruption assay, 100 μg of mitochondria were incubated with His-thioredoxin, GST-tBID, His-p53, or GST–p53 at 30 °C for 30 min. The mitochondria were pelleted at 10,000*g* for 15 min, resuspended in 1% Chaps buffer, and immunoprecipitated with an anti-Bak antibody (Upstate). Protein samples were separated by SDS–PAGE in 10% gels and analysed by western blotting using an anti-Mcl1 antibody (BD Bioscience-Pharmigen) or anti-p53 antibody (EMD Biosciences).

Treatment of Bak with trypsin. Mitochondria from H1299 or Saos2 cells were purified as described above. 15–20 μg of mitochondria were incubated with or without 25 or 125 pmol of recombinant p53 proteins, as indicated, at 30 °C for 30 min (125 pmol) or 60 min at 4 °C (25 pmol). The mitochondria were pelleted at 10,000*g* for 15 min at 4 °C and washed using fractionation buffer B without protease inhibitors. The pellets were resuspended in 25 μl of fractionation buffer B with or without trypsin (75–125 μg ml^{-1}; Invitrogen). Reaction mixtures were incubated on ice for 20 min. Trypsin digestion was stopped by adding 25 μl of 2× sample buffer (100 mM Tris-HCl at pH 6.8, 4% SDS, 0.2% bromophenol blue, 20% glycerol and 10% 2-mercaptoethanol) followed by boiling for 5 min. Reaction mixtures (10 μl) were removed from each fraction and resolved by SDS–PAGE in 4–20% gradient gels before western blotting with an anti-GRP75 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The remaining 40-μl reaction mixtures were resolved by SDS–PAGE in 4–20% gradient gels and analysed by western blotting using two different antibodies directed against the N terminus of human Bak (anti-Bak NT, Upstate USA; anti-Bak Ab-1, Oncogene Research Products, San Diego, CA).

Co-immunoprecipitation and western blotting. For *in vivo* co-immunoprecipitation studies, whole-cell extracts (500–2,000 μg) or mitochondrial lysates (50–300 μg) were prepared in 1% Chaps buffer and immunoprecipitated with the anti-Bak (Upstate), anti-Bcl-x_L (Cell Signaling Technology, Inc., Beverly, MA) or anti-Bax antibodies (Cell Signaling Technology). Precipitated proteins were washed three times in 1% Chaps buffer, loaded onto 4–20% or 10–20% Tris-glycine gradient gels, transferred overnight onto immunoblot polyvinylidene difluoride (PVDF) membranes (Bio-Rad) and detected with ECL Western Blotting Detection Reagents (Amersham Biosciences). Protein interactions were examined after solubilization of mitochondrial fractions using the zwitterionic detergent Chaps, which unlike non-ionic detergents does not induce a conformational change in Bak or Bax (refs 11, 31). For input analysis, 100 μg of whole-cell extracts or 25 μg of mitochondrial fractions lysed in 1% Chaps buffer were resolved by SDS–PAGE in 4–20% Tris-Glycine gels. Western blot analyses were performed as described previously[5,9].

Where indicated, colon carcinoma LoVo (Bax deficient) cells were pretreated with the broad-spectrum caspase inhibitor benzyloxy-carbonyl-Val-Ala-Asp-fluoromethylketone zVAD-fmk (general caspase inhibitor; BDPharmigen) at 40 μM for 30 min at 37 °C followed by treatment with or without 5 μM camptothecin for 5 h. Whole-cell extracts (5 mg) were immunoprecipitated with the anti-Bak antibody (Upstate) followed by western blotting using anti-p53 antibody (EMD Biosciences). For input analysis, 150 μg of whole-cell extracts were resolved by SDS–PAGE in 4–20% Tris-Glycine gels, followed by western blotting using anti-Bak antibody (anti-Bak NT; Upstate), anti-p53 antibody (EMD Biosciences), anti-Caspase-3 antibody (Cell Signaling Technology) and anti-PARP antibodies (EMD Biosciences).

***In vitro* mapping, *in vitro* mixing and GST-binding assays.** *In vitro* transcription-translation was performed with the TNT T7 Quick Coupled Transcription/Translation System (Promega Corporation, Madison, WI). Redivue L-^{35}S-Methionine was purchased from Amersham Biosciences (Piscataway, NJ). For *in vitro* mapping, 20 μl of ^{35}S-labelled Bak proteins were incubated with 15 μl of *in-vitro*-translated p53-R72 in 365 μl of 0.5% Chaps buffer and immunoprecipitated with 1 μg of anti-p53-antibody (AB-6; EMD Biosciences). For *in vitro* mixing experiments, 15 μl of ^{35}S-labelled Bak proteins, 45 μl of ^{35}S-labelled Bax protein or 15 μl of ^{35}S-labelled Bcl-x_L protein was incubated with 15 μl of *in-vitro*-translated p53-R72 in 1% Chaps buffer and immunoprecipitated with 1 μg anti-p53-antibody (AB-6; EMD Biosciences). Analysis of the Mcl1–Bak interaction *in vitro* was performed by mixing 15 μl of ^{35}S-labelled Mcl1 with 20 μl of wild-type or mutant Bak proteins, followed by immunoprecipitation using 1 μg of anti-Bak antibody (Upstate). After washing Protein G–agarose with 0.5% Chaps buffer three times, 30 μl of pre-washed Protein G–agarose bead slurry (15 μl of packed beads; Invitrogen) was added to each reaction, followed by gentle rocking for 30 min at 4 °C. The *in vitro* binding reactions were washed three times using 1 ml of 0.5% Chaps buffer. GST pull-down assays were performed as described[9]. Briefly, 1 μg of GST–p53 fusion proteins were incubated with 25 μl of ^{35}S-labelled full-length Bak proteins at 4 °C for 30 min with agitation. After brief centrifugation, the beads were washed five times and resuspended in protein sample buffer. Samples were subjected to SDS–PAGE. To visualize ^{35}S-labelled proteins by fluorography, SDS–PAGE gels were fixed, incubated in Amplify (Amersham Biosciences) and dried before exposure to X-ray film.

Plasmids and site-directed mutagenesis. Plasmids expressing different GST–p53 proteins were described previously[9]. The Mcl1 cDNA was generated from human placenta total RNA using the Titanium One-Step RT-PCR kit according to the manufacturer's instructions (BD Bioscience-Clontech, San Diego, CA). Full-length human Bak and Bax were obtained by PCR amplification using the pSG5-HA-Bak and pSG5-HA-Bax constructs (kindly provided by J. Marie Hardwick, The Johns Hopkins University School of Medicine, Baltimore, MD). Constructs for Bak mtBH1 (W125A, G126E, R127A), Bak mtBH2 (G175E, G176E, W177A), Bak mtBH2a (W170A, I171A, G175A, G176A) and Bak mtBH3 (L78A, D83A) were generated by site-directed mutagenesis using the QuikChange Site-Directed Mutagenesis Kit System (Stratagene, La Jolla, CA). Mutations were verified by DNA sequencing.

ACKNOWLEDGMENTS

We thank T. Lindsten and C. Thompson for the *Bak*$^{+/+}$ and *Bak*$^{-/-}$ MEFs, and J. M. Hardwick for Bak and Bax constructs. This work was supported by US Public Health Service National Institutes of Health grants CA089240, CA080854 and 5-T32-HD07516, as well as a grant from the Department of the Army (DAMD-17-02-1-0383).

COMPETING FINANCIAL INTERESTS

The authors declare that they have no competing financial interests.

Received 13 February 2004; accepted 8 March 2004
Published online at http://www.nature.com/naturecellbiology.

1. Schuler, M. & Green, D. R. Mechanisms of p53-dependent apoptosis. *Biochem. Soc. Trans.* **29,** 684–688 (2001).
2. Vousden, K. H. & Lu, X. Live or let die: the cell's response to p53. *Nature Rev. Cancer* **8,** 594–604 (2002).
3. Marchenko, N. D., Zaika, A. & Moll, U. M. Death signal-induced localization of p53 protein to mitochondria: A potential role in apoptotic signaling. *J. Biol. Chem.* **275,** 16202–16212 (2000).
4. Mihara, M. *et al.* p53 has a direct apoptogenic role at the mitochondria. *Mol. Cell* **11,** 577–590 (2003).
5. Dumont, P., Leu, J. I., Della Pietra III, A. C., George, D. L. & Murphy, M. The codon 72 polymorphic variants of p53 have markedly different apoptotic potential. *Nature Genet.* **33,** 357–365 (2003).
6. Chipuk, J. E., Maurer, U., Green, D. R. & Schuler, M. Pharmacologic activation of p53 elicits BAX-dependent apoptosis in the absence of transcription. *Cancer Cell* **4,** 371–381 (2003).
7. Beckman, G. *et al.* Is p53 polymorphism maintained by natural selection? *Hum. Hered.* **44,** 266–270 (1994).
8. Cory, S. & Adams, J. M. The Bcl2 family: regulators of the cellular life-or-death switch. *Nature Rev. Cancer* **2,** 647–656 (2002).
9. Murphy, M. *et al.* Transcriptional repression by wild-type p53 utilizes histone deacetylases, mediated by interaction with mSin3a. *Genes Dev.* **13,** 2490–2501 (1999).
10. Horikoshi, N. *et al.* Two domains of p53 interact with the TATA-binding protein, and the adenovirus 13S E1A protein disrupts the association, relieving p53-mediated transcriptional repression. *Mol. Cell. Biol.* **15,** 227–234 (1995).
11. Sattler, M. *et al.* Structure of Bcl-xL–Bak peptide complex: recognition between regulators of apoptosis. *Science* **275,** 983–986 (1997).
12. Cheng, E. H., Sheiko, T. V., Fisher, J. K., Craigen, W. J. & Korsmeyer, S. J. VDAC2 inhibits Bak activation and mitochondrial apoptosis. *Science* **301,** 513–517 (2003).
13. Griffiths, G. J. *et al.* Cell damage-induced conformational changes of the pro-apoptotic protein Bak in vivo precede the onset of apoptosis. *J. Cell Biol.* **8,** 903–914 (1999).
14. Wei, M. C. *et al.* tBID, a membrane-targeted death ligand, oligomerizes Bak to release cytochrome *c*. *Genes Dev.* **14,** 2060–2071 (2000).
15. Wei, M. C. *et al.* Proapoptotic BAX and Bak: a requisite gateway to mitochondrial dysfunction and death. *Science* **292,** 727–730 (2001).
16. Cuconati, A., Mukherjee, C., Perez, D. & White, E. DNA damage response and MCL-1 destruction initiate apoptosis in adenovirus-infected cells. *Genes Dev.* **17,** 2922–2932 (2003).
17. Ruffolo, S. C. & Shore, G. C. BCL-2 selectively interacts with the BID-induced open conformer of Bak, inhibiting Bak auto-oligomerization. *J. Biol. Chem.* **278,** 25039–25045 (2003).
18. Lindsten, T. *et al.* The combined functions of proapoptotic Bcl-2 family members bak and bax are essential for normal development of multiple tissues. *Mol Cell.* **6,** 1389–1399 (2000).
19. Cheng, E. H. *et al.* BCL-2, BCL-xL sequester BH3 domain only molecules preventing BAX- and Bak-mediated mitochondrial apoptosis. *Mol. Cell* **8,** 705–511 (2001).
20. Nijhawan, D. *et al.* Elimination of MCL-1 is required for the initiation of apoptosis following ultraviolet irradiation. *Genes Dev.* **17,** 1–12 (2003).
21. Yang, T., Buchan, H. L., Townsend, K. J. & Craig, R. W. MCL-1, a member of the BCL-2 family, is induced rapidly in response to signals for cell differentiation or death, but not to signals for cell proliferation. *J. Cell Physiol.* **166,** 523–536 (1996).
22. Bae, J., Leo, C. P., Hsu, S. Y. & Hsueh, A. J. MCL-1S, a splicing variant of the anti-apoptotic BCL-2 family member MCL-1, encodes a proapoptotic protein possessing only the BH3 domain. *J. Biol. Chem.* **275,** 25255–25261 (2000).
23. Kaufmann, S. H. *et al.* Elevated expression of the apoptotic regulator Mcl-1 at the time of leukemic relapse. *Blood* **91,** 991–1000 (1998).
24. Craig, R. W. MCL1 provides a window on the role of the BCL2 family in cell proliferation, differentiation and tumorigenesis. *Leukemia* **16,** 444–454 (2002).
25. Vrana, J. A. *et al.* An MCL1-overexpressing Burkitt lymphoma subline exhibits enhanced survival on exposure to serum deprivation, topoisomerase inhibitors, or staurosporine but remains sensitive to 1-β-D-Arabinofuranosylcytosine. *Cancer Research* **62,** 892–900 (2002).
26. Chipuk, J. E. *et al.* Direct activation of Bax by p53 mediates mitochondrial membrane permeabilization and apoptosis. *Science* **303,** 1010–1014 (2004).
27. Jimenez, G. S. *et al.* A transactivation-deficient mouse model provides insights into Trp53 regulation and function. *Nature Genet.* **26,** 37–43 (2000).
28. Pallotti, F. & Lenaz, G. Isolation and subfractionation of mitochondria from animal cells and tissue culture lines. *Methods Cell Biol.* **65,** 1–35 (2001).
29. Bessette, P. H., Aslund, F., Beckwith, J. & Georgiou, G. Efficient folding of proteins with multiple disulfide bonds in Escherichia coli cytoplasm. *Proc. Natl Acad. Sci. USA* **96,** 13703–13708 (1999).
30. Hsu, Y. T. & Youle, R. J. Bax in murine thymus is a soluble monomeric protein that displays differential detergent-induced conformations. *J. Biol. Chem.* **273,** 10777–10783 (1998).

[Cell Cycle 3:7, 836-839; July 2004]; ©2004 Landes Bioscience

Extra Views

p53 Moves to Mitochondria

A Turn on the Path to Apoptosis

Maureen E. Murphy[1]

J. I-Ju Leu[2]

Donna L. George[2,*]

[1]Department of Pharmacology; Fox Chase Cancer Center; Philadelphia; Pennsylvania USA

[2]Department of Genetics; University of Pennsylvania School of Medicine; Philadelphia, Pennsylvania USA

*Correspondence to: Donna L. George; Department of Genetics; University of Pennsylvania School of Medicine; 475 Clinical Research Building; 415 Curie Blvd.; Philadelphia, Pennsylvania USA 19104; Tel: 215.898.5032; Fax: 215.573.9411; Email: georged@mail.med.upenn.edu

Received 05/03/04; Accepted 05/04/04

Previously published online as a *Cell Cycle* E-Publication:
http://www.landesbioscience.com/journals/cc/abstract.php?id=956

KEY WORDS

p53, BAK, MCL1, apoptosis, mitochondria, polymorphism, tumor suppressor

ACKNOWLEDGEMENTS

This work was supported by NIH grants CA89240 and CA80854 as well as a grant from the Department of the Army, DAMD-17-02-1-0383.

[Cell Cycle 3:7, 836-839; July 2004]; ©2004 Landes Bioscience

Extra Views

p53 Moves to Mitochondria

A Turn on the Path to Apoptosis

Maureen E. Murphy[1]
J. I-Ju Leu[2]
Donna L. George[2,*]

[1]Department of Pharmacology; Fox Chase Cancer Center; Philadelphia; Pennsylvania USA

[2]Department of Genetics; University of Pennsylvania School of Medicine; Philadelphia, Pennsylvania USA

*Correspondence to: Donna L. George; Department of Genetics; University of Pennsylvania School of Medicine; 475 Clinical Research Building; 415 Curie Blvd.; Philadelphia, Pennsylvania USA 19104; Tel: 215.898.5032; Fax: 215.573.9411; Email: georged@mail.med.upenn.edu

Received 05/03/04; Accepted 05/04/04

Previously published online as a *Cell Cycle* E-Publication: http://www.landesbioscience.com/journals/cc/abstract.php?id=956

KEY WORDS

p53, BAK, MCL1, apoptosis, mitochondria, polymorphism, tumor suppressor

ACKNOWLEDGEMENTS

This work was supported by NIH grants CA89240 and CA80854 as well as a grant from the Department of the Army, DAMD-17-02-1-0383.

ABSTRACT

It has been said that no matter which direction cancer research turns, the p53 tumor suppressor protein comes into view. The widespread role of p53 as a suppressor of tumor development is believed to rely on its ability to induce programmed cell death in response to stress, either the replicative stress associated with uncontrolled cellular proliferation, or the environmental stresses that accompany tumor development, such as hypoxia. For some time it has been believed that the role of p53 in inducing apoptosis in response to such stress was as a master regulator coordinating the expression of other molecules whose ultimate role was the execution of the cell. New data, however, suggest that p53 itself also has a direct role in accomplishing cell death, at the mitochondria.

Since its discovery a quarter century ago, the p53 tumor suppressor protein has been recognized as a key factor in the induction of programmed cell death and cell cycle checkpoint control in response to genotoxic and environmental stress.[1-3] The p53 pathway is inactivated in the majority of human cancers, most likely because the pro-apoptotic function of p53 is critical to the inhibition of tumor development and progression. Thus, the past twenty-five years have seen intensive and varied investigations to better understand the functions that p53 uses to mediate apoptosis. Clearly established is p53's role as a nuclear transcription factor with the ability to activate, or repress, the expression of many genes.[1-5] A number of p53 transcriptional targets, such as the p53-induced genes *BAX*, *PUMA*, *NOXA*, and the p53-repressed genes *BCL2* and *SURVIVIN*, represent genes with the potential to promote or inhibit apoptosis, respectively, in stressed cells. More recently, however, a somewhat unexpected turn in the p53-mediated pathway to programmed cell death has emerged with accumulating data that p53 has a direct cytoplasmic role at mitochondria in activating the apoptotic machinery.[6-11]

Because others[2,3,6,7,9,12] have expertly summarized the evidence supporting a transcription-independent function of p53 in apoptosis, that information will not be repeated here. Rather, we will touch briefly on a few points to exemplify the phenomenon. It has been reported, for example, that under some circumstances, p53-dependent apoptosis can occur in the absence of new protein synthesis, thereby excluding p53's function as a transcriptional activator.[13,14] Also, certain transcriptionally inactive mutants of p53 can still induce apoptosis when overexpressed in tumor cells.[15,16] Finally, in response to some stresses, such as hypoxia, p53 induces apoptosis but does not function as a transactivator.[17] Intriguingly, Moll and colleagues demonstrated that during p53-dependent apoptosis a fraction of cellular p53 protein localizes to mitochondria and induces cytochrome c release; however, this is not observed during p53-mediated cell cycle arrest.[6] Additional support for the concept that p53 has a cytoplasmic role in apoptosis induction resulted from our functional analysis of polymorphic variants of p53.[8]

Within exon 4 of the p53 gene, a common single-nucleotide polymorphism (SNP) at codon 72 leads to the incorporation of either an arginine (R72) or a proline (P72) at this position of the protein. This SNP was first noted more than twenty years ago as a non-tumor derived amino acid difference that affected the mobility of p53 on SDS-polyacrylamide gels.[18,19] This observation raised the possibility that this particular amino acid change might affect the structure of the p53 protein and, perhaps, its biological activities as well. The codon 72 polymorphism is maintained at different allele frequencies in different populations, and there appears to be a selection against the R72 allele in populations living near the equator.[20] These and other studies indicated that functional differences between the R72 and P72 variants might exist.[20-22] We further investigated this possibility using cell lines containing inducible versions of alleles encoding the R72 and P72 protein forms, as well as in human cell lines expressing endogenous p53. Our studies revealed that the

R72 form of p53 induces apoptosis markedly better than the P72 variant.[8] A similar conclusion has also been reached in two recent publications.[23,24] When we explored the potential mechanisms underlying the observed functional difference between the two p53 variants, we made the initially surprising discovery that the greater apoptotic potential of the R72 form correlated with its much better ability to traffic to mitochondria. We showed that this enhanced mitochondrial localization of the R72 protein was associated with increased nuclear export, due to increased binding of R72 to MDM2, which catalyzes nuclear export of p53.[8] Based on these data, therefore, we concluded that the enhanced apoptosis-inducing activity of the R72 protein related, at least in part, to its greater mitochondrial localization.

A major question arising from that study centered on defining the apoptotic function of mitochondrial p53. We reasoned that mechanistic insight to this process could be obtained from the identification of mitochondrial p53-interacting proteins. Using affinity chromatography protocols and mass spectrometry, we have now uncovered a direct interaction between the p53 tumor suppressor protein and the mitochondrial death-effector protein BAK.[11] BAK is a pro-apoptotic member of the BCL2-family of proteins.[25,26] An analysis of whole cell or mitochondrial extracts by immunoprecipitation-western blot analysis, demonstrated that the R72 form of p53 binds better to BAK than does the P72 variant, correlating with the differences in apoptotic potential of the two p53 variants. Interestingly, however, our in vitro analyses revealed that the R72 and P72 proteins bind equally well to BAK. These observations are consistent with the conclusion that the enhanced interaction between BAK and the R72 variant observed in intact cells reflects the enhanced nuclear export and mitochondrial trafficking of this p53 isoform.

In healthy cells, BAK resides at mitochondria as an inactive monomer. In response to various death stimuli, it undergoes an activating allosteric conformational change that promotes homo-oligomerization. This leads to formation of a pore in the outer mitochondrial membrane, and allows the release of cytochrome c and other pro-apoptogenic factors from the mitochondria resulting in the activation of a caspase cascade.[27-29] Having established that stress-activated p53 physically interacts with BAK, we then sought to test whether p53 has the ability to conformationally alter BAK and mediate the release of cytochrome c from mitochondria. In well-established assays utilizing purified mitochondria, we determined that p53 behaved in a manner similar to that of another BAK-interacting protein, tBID, which also catalyzes the oligomerization of BAK.[28] Specifically, incubation of mitochondria with even nanomolar amounts of recombinant p53 rapidly induced a conformational change in BAK, along with BAK oligomerization and cytochrome c release. Importantly, we showed that p53 requires the presence of BAK on purified mitochondria in order to induce cytochrome c release, indicating that p53 is acting through a previously recognized genetic pathway of mitochondrial apoptosis.

Given the discovery that p53 can bind and activate BAK, we sought to elucidate how this interaction might impact on other key factors that regulate the programmed cell death machinery. Reasoning that p53 might alter the interaction of BAK with anti-apoptotic BCL-2 family members, we focused attention on another mitochondrial protein, MCL1. This decision was based, in part, on previous observations that anti-apoptotic proteins such as BCL-2 and BCL-X_L are not normally present at the mitochondria and do not appreciably interact with BAK in unstressed cells.[30-32]

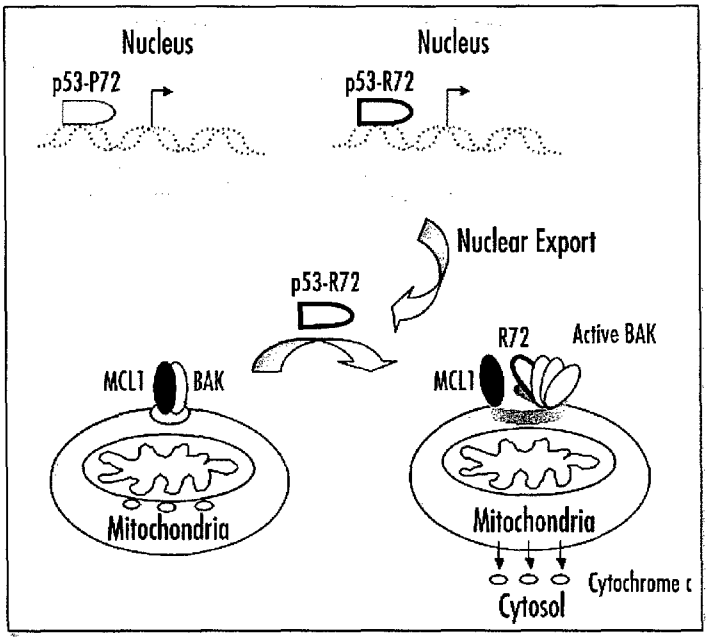

Figure 1. A model for the role of mitochondrial p53. In response to cell stress signals, p53 is activated. Relative to the P72 form of p53, the R72 variant exhibits a greater ability to traffic to mitochondria. Binding of the R72 variant to pro-apoptotic mitochondrial membrane protein BAK leads to disruption of BAK-MCL1 interaction. The formation of a p53-BAK complex induces a conformational change in BAK, BAK oligomerization, and the release of cytochrome c from mitochondria to the cytosol.

Even more interesting, however, were recent data[32] indicating that this anti-apoptotic BCL-2 family member, MCL1, functions as a critical upstream regulator of the mitochondrial apoptotic program. The downregulation or loss of MCL1 seems to be necessary, albeit insufficient, to initiate the cell death process, preceding events such as BAX/BCL-X_L translocation to mitochondria and BAK oligomerization. In this regard, Cuconati et al. have found that in unstressed cells, BAK is complexed with MCL1, and this interaction is lost following adenovirus E1A-initiated apoptosis.[33] We determined that MCL1 is complexed with BAK in normal cells, but that the stress-induced activation of p53 leads to disruption of the BAK-MCL1 complex.[11] Additionally, we demonstrated that formation of a p53-BAK complex and concomitant disruption of the BAK-MCL1 interaction represent early events in apoptosis that occur prior to cytochrome c release and caspase activation. Collectively, then, these observations support a model in which the tumor suppressor p53 and the anti-apoptotic MCL1 protein have opposing upstream roles in mitochondrial apoptosis: by directly binding to mitochondrial BAK, the MCL1 and p53 proteins function either to inhibit or to promote, respectively, BAK's pro-apoptotic behavior (Fig. 1).

The data described above provide new insight into the mechanisms involved in an extra-nuclear direct role of p53 in apoptosis induction, binding and oligomerization of BAK. Recently, like BAK, the BCL2 family members BAX and BCL-X_L have also been implicated in mitochondrial apoptosis induction by p53.[9,10] Employing both in vivo and in vitro binding assays, we have detected much less, if any, association between these proteins and p53. These discrepant results may stem from variations in experimental conditions used by different groups; indeed, binding conditions must be stringently controlled in such assays, as the conformation and binding activity

of BCL-2 family proteins, including BAX and BCL-X_L, are known to be strongly influenced by buffer and detergent conditions.[31,34] The biological importance of a p53-BAK interaction is supported by the finding that p53 can efficiently cause cytochrome c release from isolated mitochondria even if these organelles contain little if any BAX,[9] but cannot do so if these organelles are BAK-deficient.[11] Furthermore, tumor cells deficient in BAX, or BCL-X_L, are still competent for p53-dependent apoptosis associated with the formation of a p53-BAK complex at mitochondria (our unpublished observations).

The combined data argue for a direct role of p53 in programmed cell death. However, if one is to accept the relevance and significance of the mitochondrial pathway of p53-dependent apoptosis, at least two findings in the literature must be explained. The first is the finding by Wahl and colleagues that a synthetic p53 mutant that is transcriptionally inactive (p53QS) is also completely unable to function as a tumor suppressor in genetically-engineered mice.[35] Additionally, mice that are genetically engineered to lack the BH3-only protein PUMA, a transcriptional target of p53 and BCL-2 family member, have cells that are greatly impaired for apoptosis induction.[36] These two findings have led to the notion that the transcriptional activity of p53 is largely, if not wholly, responsible for apoptosis induction by this protein. Such a conclusion would be not be supported by the existent data, however. For example, the transcriptionally-inactive p53QS mutant is also known to be unable to bind to MDM2.[35,37] It has been shown that MDM2 is essential for efficient nuclear export of p53,[38,39] and further that forms of p53 that interact less well with MDM2, like the proline 72 polymorphic variant, also exhibit impaired mitochondrial localization.[8] In line with this, Wahl and colleagues reported[35] that the p53QS mutant had greatly impaired nuclear export, and abnormal retention of nuclear p53; therefore, this synthetic mutant would be predicted to have a dysfunctional mitochondrial pathway. Interestingly, the p53 target gene PUMA is also a component of the mitochondrial apoptosis pathway; it has been proposed that PUMA induces cell death by inhibiting the activity of anti-apoptotic BCL-2 family members.[40,41] Therefore, it will be of interest to determine if the loss of PUMA alters the threshold of active BAK at the mitochondria, thereby impairing oligomerization of BAK by p53. This hypothesis remains to be tested.

While some questions about the actions of mitochondrial p53 have now been answered, many others remain. Certainly a key issue centers on defining the relative contribution of the mitochondrial function of p53 to overall p53-mediated cellular apoptosis. Also, it remains to be determined if this extra-nuclear function of p53 is preferentially activated in tumor cells, relative to normal cells, and if there are other factors (including cell type or nature of the stress) involved in targeting p53 to mitochondria. It is of interest that the stress-induced localization of p53 to mitochondria has been observed in most tumor cells and rapidly proliferating normal cells, but not in cells that tend to growth arrest in response to p53, such as human fibroblasts.[6,8] These findings raise the possibility that the mitochondrial localization of p53 may be the elusive deciding factor that dictates whether cells die in response to p53, or whether they growth arrest. Another factor that should be considered in evaluating the literature and in designing future studies in this area concerns the codon 72 polymorphism of p53. As discussed above, this common polymorphism strongly influences the degree of p53 mitochondrial localization and apoptosis.[8] Recent data also suggest that it is an important determinant of the response to chemotherapeutic agents in tumor cells with wild type p53.[24]

The loss of p53 function directly contributes to tumor progression and chemoresistance. Thus, restoring key elements of this pathway represents an obvious and attractive target for cancer diagnosis and therapeutics. Approaching this goal, however, requires a clearer understanding of the mechanisms by which p53 acts in the execution of cell death pathways. The studies summarized above strengthen the conclusion that p53 can function directly at the mitochondria to promote apoptosis, and provide the groundwork for a novel therapeutic pathway.

References

1. Vousden KH, Lu X. Live or let die: The cell's response to p53. Nature Rev Cancer 2002; 8:594-604.
2. Friedman JS, Lowe SW. Control of apoptosis by p53. Oncogene 2003; 22:9030-40.
3. Oren M. Decision making by p53: Life, death and cancer. Cell Death Differ 2003; 10:431-42.
4. Schuler M, Green DR. Mechanisms of p53-dependent apoptosis. Biochem Soc Trans 2001; 29:684-88.
5. Ho J, Benchimol S. Transcriptional repression mediated by the p53 tumor suppressor. Cell Death Differ 2003; 10:404-8.
6. Marchenko ND, Zaika A, Moll UM. Death signal-induced localization of p53 protein to mitochondria: A potential role in apoptotic signaling. J Biol Chem 2000; 275:16202-12.
7. Chipuk JE, Maurer U, Green DR, Schuler M. Pharmacologic activation of p53 elicits BAX-dependent apoptosis in the absence of transcription. Cancer Cell 2003; 4:371-81.
8. Dumont P, Leu JI, Della Pietra III AC, George DL, Murphy M. The codon 72 polymorphic variants of p53 have markedly different apoptotic potential. Nature Genetics 2003; 33:357-65.
9. Mihara M, Erster S, Zaika A, Petrenko O, Chitenden T, Pancoska P, et al. p53 has a direct apoptogenic role at the mitochondria. Mol Cell 2003; 11:577-90.
10. Chipuk JE, Kuwana T, Bouchier-Hayes L, Droin NM, Newmeyer DD, Shuler M, et al. Direct activation of Bax by p53 mediates membrane permeabilization and apoptosis. Science 2004; 303:1010-14.
11. Leu JI-J, Dumont P, Hafey M, Murphy ME, George DL. Mitochondrial p53 activates BAK and leads to disruption of a BAK-MCL1 complex. Nature Cell Biol 2004; 6:443-50.
12. Moll UM, Zaika A. Nuclear and mitochondrial apoptotic pathways of p53. FEBS Lett 2001; 493:65-9.
13. Caelles C, Hellmberg A, Karin M. p53-dependent apoptosis in the absence of transcriptional activation of p53 targets. Nature 1994; 370:220-23.
14. Wagner AJ, Kokontis JM, Hay N. Myc-mediated apoptosis requires wild type p53 in a manner independent of cel cycle arrest and the ability of p53 to induce p21wafl/cip1. Genes Dev 1994; 8:2817-30.
15. Haupt Y, Rowan S, Shaulian E, Vousden KH, Oren M. Induction of apoptosis in HeLa cells by trans-activation-deficient p53. Genes Dev 1995; 9:2170-83.
16. Chen X, Ko LJ, Jayaraman L, Prives C. p53 levels, functional domains, and DNA damage determine the extent of the apoptotic response of tumor cells. Genes Dev 1996; 10:2438-51.
17. Koumenis C, Alarcon R, Hammond E, Sutphin P, Hoffman W, Murphy M, et al. Regulation of p53 by hypoxia: Dissociation of transcriptional repression and apoptosis from p53 -dependent transactivation. Mol Cell Biol 2001; 21:1297-310.
18. Harris N, Brill E, Shohat O, Prokicimer M, Wolf D, Arai N, et al. Molecular basis for heterogenetiy of the human p553 protein. Mol Cell Biol 1986; 6:4650-6.
19. Matlashewski GJ, Tuck S, Pim D, Lamb P, Schneider J, Crawford LV. Primary structure polymorphism at amino acid residue 72 of human p53. Mol Cell Biol 1987; 7:961-3.
20. Beckman G, Birgander R, Sjalander A, Saha N, Holmberg PA, Kivela A, et al. Is p53 polymorphism maintained by natural selection? Hum Hered 1994; 44:266-70.
21. Storey A, Thomas M, Kalita A, Harwood C, Gardiol D, Mantovani F, et al. Role of a p53 polymorphism in the development of human papilloma-virus-associated cancer. Nature 1998; 393:229-34.
22. Thomas M, Kalita A, Labrecque S, Pim D, Banks L, Matlashewki G. Two polymorphic variants of wild-type p53 differ biochemically and biologically. Mol Cell Biol 1999; 19:1092-100.
23. Pim D, Banks L. p53 polymorphic variants at codon 72 exert different effects on cell cycle progression. Int J Cancer 2004; 108:196-9.
24. Sullivan A, Syed N, Gasco M, Bergamaschi D, Trigiante G, Attard M, et al. Polymorphism in wild-type p53 modulates response to chemotherapy in vitro and in vivo. Oncogene 2004; 23:3328-37.
25. Gross A, McDonnell JM, Korsmeyer SJ. BCL-2 family members and the mitochondria in apoptosis. Genes Dev 1999; 13:1899-911.
26. Cory S, Adams JM. The Bcl2 family: Regulators of the cellular life-or-death switch. Nat Rev Cancer 2002; 2:647-56.
27. Griffiths GJ, Dubrez L, Morgan CP, Jones NA, Whitehouse J, Corfe BM, et al. Cell damage-induced conformational changes of the pro-apoptotic protein BAK in vivo precede the onset of apoptosis. J Cell Biol 1999; 8:903-14.
28. Wei MC, Lindsten T, Mootha VK, Weiler S, Gross A, Ashiya M, et al. tBID, a membrane-targeted death ligand, oligomerizes BAK to release cytochrome c. Genes Dev 2000; 14:2060-71.

29. Wei MC, Zong WX, Cheng EH, Lindsten T, Panoutsakopoulou V, Ross AJ, et al. Proapoptotic BAX and BAK: A requisite gateway to mitochondrial dysfunction and death. Science 2001; 292:727-30.
30. Cheng EH, Wei M, Weiler S, Flavell R, Mak T, Lindsten T, et al. BCL-2, BCL-xL sequester BH3 domain only molecules preventing BAX- and BAK-mediated mitochondrial apoptosis. Mol Cell 2001; 8:705-11.
31. Cheng EH, Sheiko TV, Fisher JK, Craigen WJ, Korsmeyer SJ. VDAC2 inhibits BAK activation and mitochondrial apoptosis. Science 2003; 301:513-7.
32. Nijhawan D, Fang M, Traer E, Zhong Q, Gao W, Du F, et al. Elimination of MCL-1 is required for the initiation of apoptosis following ultraviolet irradiation. Genes Dev 2003; 17:1475-86.
33. Cuconati A, Mukherjee C, Perez D, White E. DNA damage response and MCL-1 destruction initiate apoptosis in adenovirus-infected cells. Genes Dev 2003; 17:2922-32.
34. Hsu YT, Youle RJ. Bax in murine thymus is a soluble monomeric protein that displays differential detergent-induced conformations. J Biol Chem 1998; 273:10777-83.
35. Jimenez GS, Nister M, Stommel JM, Beeche M, Barcarse EA, Zhang X-Q, et al. A transactivation-deficient mouse model provides insights into Trp53 regulation and function. Nat Genet 2000; 26:37-43.
36. Jeffers JR, Parganas E, Lee Y, Yang C, Wang JL, Brennan J, et al. Puma is an essential mediator of p53-dependent and -independent apoptotic pathways. Cancer Cell 2003; 4:321-28.
37. Lin J, Chen J, Elenbass B, Levine AJ. Several hydrophobic amino acids in the p53 amino-terminal domain are required for transcriptional activation, binding to mdm-2 and the adenovirus5 E1B 55-kD protein. Genes Dev 1994; 8:1235-46.
38. Boyd SD, Tsai KY, Jacks T. An intact HDM2 RING-finger domain is required for nuclear exclusion of p53. Nat Cell Biol 2000; 2:563-8.
39. Geyer RK, Yu ZK, Maki CG. The MDM2 RING-finger domain is required to promote p53 nculear export. Nat Cell Biol 2000; 569-73.
40. Nakano K, Vousdent KH. PUMA, a novel proapoptotic gene, is induced by p53. Mol Cell 2001; 7:683-94.
41. Zhang JY, Hwang PM, Kinzler KW, Vogelstein B. PUMA induces the rapid apoptosis of colorectal cancer cells. Mol Cell 2004; 7:673-82.